(51) International Patent Classification 5 : C12N 15/13, C12P 21/08 C07K 13/00, C12N 5/10 G06F 15/00	A1	(11) International Publication Number: WO 94/04679
		(43) International Publication Date: 3 March 1994 (03.03.94)

(54) Title: METHOD FOR MAKING HUMANIZED ANTIBODIES

(57) Abstract

Variant immunoglobulins, particularly humanized antibody polypeptides are provided, along with methods for their preparation and use. Consensus immunoglobulin sequences and structural models are also provided.

METHOD FOR MAKING HUMANIZED ANTIBODIES

Field f the Invention

This invention relates to methods for the preparation and use of variant antibodies and

5   finds application particularly in the fields of immunology and cancer diagnosis and therapy.

Background of the Invention

Naturally occurring antibodies (immunoglobulins) comprise two heavy chains linked together by disulfide bonds and two light chains, one light chain being linked to each of the

10   heavy chains by disulfide bonds. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain (V_L) at one end and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are

15   believed to form an interface between the light and heavy chain variable domains, see e.g. Chothia et al., J. Mol. Biol. 186:651-663 (1985); Novotny and Haber, Proc. Natl. Acad. Sci. USA 82:4592-4596 (1985).

The constant domains are not involved directly in binding the antibody to an antigen, but are involved in various effector functions, such as participation of the antibody in

20   antibody-dependent cellular cytotoxicity. The variable domains of each pair of light and heavy chains are involved directly in binding the antibody to the antigen. The domains of natural light and heavy chains have the same general structure, and each domain comprises four framework (FR) regions, whose sequences are somewhat conserved, connected by three hyper-variable or complementarity determining regions (CDRs) (see Kabat, E. A. et al.,

25   Sequences of Proteins of Immunological Interest, National Institutes of Health, Bethesda, MD, (1987)). The four framework regions largely adopt a β-sheet conformation and the CDRs form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held in close proximity by the framework regions and, with the CDRs from the other chain, contribute to the formation of the antigen binding site.

30   Widespread use has been made of monoclonal antibodies, particularly those derived from rodents including mice, however they are frequently antigenic in human clinical use. For example, a major limitation in the clinical use of rodent monoclonal antibodies is an anti-globulin response during therapy (Miller, R. A. et al., Blood 62:988-995 (1983); Schroff, R. W. et al., Cancer Res. 45:879-885 (1985)).

35   The art has attempted to overcome this problem by constructing "chimeric" antibodies in which an animal antigen-binding variable domain is coupled to a human constant domain (Cabilly et al., U.S. patent No. 4,816,567; Morrison, S. L. et al., Proc. Natl. Acad. Sci. USA 81:6851-6855 (1984); Boulianne, G. L. et al., Nature 312:643-646 (1984); Neuberger, M.

S. et al., Nature 314:268-270 (1985)). The term "chimeric" antib dy is used her in to describe a polypeptide c mprising at least the antigen binding porti n of an antibody molecule linked to at l ast part of another protein (typically an immunoglobulin constant domain).

5   The isotype of the human constant domain may be sel cted to tail r the chimeric antibody for participation in antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (see e.g. Brüggemann, M. et al., J. Exp. Med. 166:1351-1361 (1987); Riechmann, L. et al., Nature 332:323-327 (1988); Love et al., Methods in Enzymology 178:515-527 (1989); Bindon et al., J. Exp. Med. 168:127-142 (1988).

10   In the typical embodiment, such chimeric antibodies contain about one third rodent (or other non-human species) sequence and thus are capable of eliciting a significant anti-globulin response in humans. For example, in the case of the murine anti-CD3 antibody, OKT3, much of the resulting anti-globulin response is directed against the variable region rather than the constant region (Jaffers, G. J. et al., Transplantation 41:572-578 (1986)).

15   In a further effort to resolve the antigen binding functions of antibodies and to minimize the use of heterologous sequences in human antibodies, Winter and colleagues (Jones, P. T. et al., Nature 321:522-525 (1986); Riechmann, L. et al., Nature 332:323-327 (1988); Verhoeyen, M. et al., Science 239:1534-1536 (1988)) have substituted rodent CDRs or CDR sequences for the corresponding segments of a human antibody. As used herein, the term

20   "humanized" antibody is an embodiment of chimeric antibodies wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

25   The therapeutic promise of this approach is supported by the clinical efficacy of a humanized antibody specific for the CAMPATH-1 antigen with two non-Hodgkin lymphoma patients, one of whom had previously developed an anti-globulin response to the parental rat antibody (Riechmann, L. et al., Nature 332:323-327 (1988); Hale, G. et al., Lancet I:1394-1399 (1988)). A murine antibody to the interleukin 2 receptor has also recently been

30   humanized (Queen, C. et al., Proc. Natl. Acad. Sci. USA 86:10029-10033 (1989)) as a potential immunosuppressive reagent. Additional references related to humanization of antibodies include Co et al., Proc. Natl. Acad. Sci. USA 88:2869-2873 (1991); Gorman et al., Proc. Natl. Acad. Sci. USA 88:4181-4185 (1991); Daugherty et al., Nucleic Acids Research 19(9):2471-2476 (1991); Brown et al., Proc. Natl. Acad. Sci. USA 88:2663-2667 (1991);

35   Junghans et al., Cancer Research 50:1495-1502 (1990).

In some cases, substituting CDRs from rodent antibodies for the human CDRs in human frameworks is sufficient to transfer high antigen binding affinity (Jones, P. T. et al., Nature 321:522-525 (1986); Verhoeyen, M. et al., Science 239:1534-1536 (1988)), whereas in

other ases it has been necessary t additionally replace ne (Riechmann, L. *et al., Nature* 332:323-327 (1988)) or several (Queen, C. *et al., Proc. Natl. Acad. Sci. USA* 86:10029-10033 (1989)) framework region (FR) residues. See also Co *et al., supra.*

For a given antibody a small number of FR residues are anticipated to be important for

5   antigen binding. Firstly for example, certain antibodies have been shown to contain a few FR residues which directly contact antigen in crystal structures of antibody-antigen complexes (e.g., reviewed in Davies, D. R. *et al., Ann. Rev. Biochem.* 59:439-473 (1990)). Secondly, a number of FR residues have been proposed by Chothia, Lesk and colleagues (Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901-917 (1987); Chothia, C. *et al., Nature* 342:877-883

10   (1989); Tramontano, A. *et al., J. Mol. Biol.* 215:175-182 (1990)) as critically affecting the conformation of particular CDRs and thus their contribution to antigen binding. See also Margolies *et al., Proc. Natl. Acad. Sci. USA* 72:2180-2184 (1975).

It is also known that, in a few instances, an antibody variable domain (either V_H or V_L) may contain glycosylation sites, and that this glycosylation may improve or abolish antigen

15   binding, Pluckthun, *Biotechnology* 9:545-51 (1991); Spiegelberg *et al., Biochemistry* 9:4217-4223 (1970); Wallic *et al., J. Exp. Med.* 168:1099-1109 (1988); Sox *et al., Proc. Natl. Acad. Sci. USA* 66:975-982 (1970); Margni *et al., Ann. Rev. Immunol.* 6:535-554 (1988). Ordinarily, however, glycosylation has no influence on the antigen-binding properties of an antibody, Pluckthun, *supra,* (1991).

20   The three-dimensional structure of immunoglobulin chains has been studied, and crystal structures for intact immunoglobulins, for a variety of immunoglobulin fragments, and for antibody-antigen complexes have been published (see e.g., Saul *et al., Journal of Biological Chemistry* 25:585-97 (1978); Sheriff *et al., Proc. Natl. Acad. Sci. USA* 84:8075-79 (1987); Segal *et al., Proc. Natl. Acad. Sci. USA* 71:4298-4302 (1974); Epp *et al., Biochemistry*

25   14(22):4943-4952 (1975); Marquart *et al., J. Mol. Biol.* 141:369-391 (1980); Furey *et al., J. Mol. Biol.* 167:661-692 (1983); Snow and Amzel, *Protein: Structure, Function, and Genetics* 1:267-279, Alan R. Liss, Inc. pubs. (1986); Chothia and Lesk, *J. Mol. Biol.* 196:901-917 (1987); Chothia *et al., Nature* 342:877-883 (1989); Chothia *et al., Science* 233:755-58 (1986); Huber *et al., Nature* 264:415-420 (1976); Bruccoleri *et al., Nature* 335:564-568

30   (1988) and *Nature* 336:266 (1988); Sherman *et al., Journal of Biological Chemistry* 263:4064-4074 (1988); Amzel and Poljak, *Ann. Rev. Biochem.* 48:961-67 (1979); Silverton *et al., Proc. Natl. Acad. Sci. USA* 74:5140-5144 (1977); and Gregory *et al., Molecular Immunology* 24:821-829 (1987). It is known that the function of an antibody is dependent on its three dimensional structure, and that amino acid substitutions can change the three-

35   dimensional structure of an antibody, Snow and Amzel, *supra.* It has previously been shown that the antigen binding affinity of a humanized antibody can be increased by mutagenesis based upon molecular modelling (Riechmann, L. *et al., Nature* 332:323-327 (1988); Queen, C. *et al., Proc. Natl. Acad. Sci. USA* 86:10029-10033 (1989)).

Humanizing an antibody with retention of high affinity for antigen and other desired biological activities is at present difficult to achieve using currently available procedures. Methods are needed for rationalizing the selection of sites for substitution in preparing such antibodies and thereby increasing the efficiency of antibody humanization.

The proto-oncogene *HER2* (human epidermal growth factor receptor 2) encodes a protein tyrosine kinase (p185^{HER2}) that is related to and somewhat homologous to the human epidermal growth factor receptor (see Coussens, L. et al., *Science* 230:1132-1139 (1985); Yamamoto, T. et al., *Nature* 319:230-234 (1986); King, C. R. et al., *Science* 229:974-976 (1985)). *HER2* is also known in the field as *c-erbB-2*, and sometimes by the name of the rat homolog, *neu*. Amplification and/or overexpression of *HER2* is associated with multiple human malignancies and appears to be integrally involved in progression of 25-30% of human breast and ovarian cancers (Slamon, D. J. et al., *Science* 235:177-182 (1987), Slamon, D. J. et al., *Science* 244:707-712 (1989)). Furthermore, the extent of amplification is inversely correlated with the observed median patient survival time (Slamon, *supra*, Science 1989).

The murine monoclonal antibody known as muMAb4D5 (Fendly, B. M. et al., *Cancer Res.* 50:1550-1558 (1990)), directed against the extracellular domain (ECD) of p185^{HER2}, specifically inhibits the growth of tumor cell lines overexpressing p185^{HER2} in monolayer culture or in soft agar (Hudziak, R. M. et al., *Molec. Cell. Biol.* 9:1165-1172 (1989); Lupu, R. et al., *Science* 249:1552-1555 (1990)). MuMAb4D5 also has the potential of enhancing tumor cell sensitivity to tumor necrosis factor, an important effector molecule in macrophage-mediated tumor cell cytotoxicity (Hudziak, *supra*, 1989; Shepard, H. M. and Lewis, G. D. *J. Clinical Immunology* 8:333-395 (1988)). Thus muMAb4D5 has potential for clinical intervention in and imaging of carcinomas in which p185^{HER2} is overexpressed. The muMAb4D5 and its uses are described in PCT application WO 89/06692 published 27 July 1989. This murine antibody was deposited with the ATCC and designated ATCC CRL 10463. However, this antibody may be immunogenic in humans.

It is therefore an object of this invention to provide methods for the preparation of antibodies which are less antigenic in humans than non-human antibodies but have desired antigen binding and other characteristics and activities.

It is a further object of this invention to provide methods for the efficient humanization of antibodies, i.e. selecting non-human amino acid residues for importation into a human antibody background sequence in such a fashion as to retain or improve the affinity of the non-human donor antibody for a given antigen.

It is another object of this invention to provide humanized antibodies capable of binding p185^{HER2}.

Other objects, features, and characteristics of the present invention will become apparent upon consideration of the following description and the appended claims.

Summary of the Invention

The bjects of this invention are acc mplished by a method for making a humanized antibody comprising amino acid s quence of an imp rt, n n-human antibody and a human antibody, comprising the steps of:

5   a.   obtaining the amino acid sequences of at least a portion of an import antibody variable domain and of a consensus variable domain;

b.   identifying Complementarity Determining Region (CDR) amino acid sequences in the import and the human variable domain sequences;

c.   substituting an import CDR amino acid sequence for the corresponding human
10   CDR amino acid sequence;

d.   aligning the amino acid sequences of a Framework Region (FR) of the import antibody and the corresponding FR of the consensus antibody;

e.   identifying import antibody FR residues in the aligned FR sequences that are non-homologous to the corresponding consensus antibody residues;

15   f.   determining if the non-homologous import amino acid residue is reasonably expected to have at least one of the following effects:

1.   non-covalently binds antigen directly,

2.   Interacts with a CDR; or

3.   participates in the V_L - V_H interface; and

20   g.   for any non-homologous import antibody amino acid residue which is reasonably expected to have at least one of these effects, substituting that residue for the corresponding amino acid residue in the consensus antibody FR sequence.

Optionally, the method of this invention comprises the additional steps of determining if any non-homologous residues identified in step (e) are exposed on the surface of the
25 domain or buried within it, and if the residue is exposed but has none of the effects identified in step (f), retaining the consensus residue.

Additionally, in certain embodiments the method of this invention comprises the feature wherein the corresponding consensus antibody residues identified in step (e) above are selected from the group consisting of 4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L,
30 65L, 66L, 67L, 68L, 69L, 70L, 71L, 73L, 85L, 87L, 98L, 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H (utilizing the numbering system set forth in Kabat, E. A. *et al., Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987)).

In certain embodiments, the method of this invention comprises the additional steps of
35 searching either or both of the import, non-human and the consensus variable domain sequences for glycosylation sites, determining if the glycosylation is reasonably expected to be important for the desired antigen binding and biological activity of the antibody (i.e., determining if the glycosylation site binds to antigen or changes a side chain of an amino acid

residue that binds t antigen, or if the glycosylation enhances or weakens antigen binding, or is important for maintaining antib dy affinity). If the import sequence bears the glyc sylation site, it is preferred to substitute that site for the corresponding residues in the consensus human if the glycosylation site is reasonably expected to be important. If only the consensus

5   sequence, and not the import, bears the glycosylation site, it is preferred to eliminate that glycosylation site or substitute therefor the corresponding amino acid residues from the import sequence.

Another embodiment of this invention comprises aligning import antibody and the consensus antibody FR sequences, identifying import antibody FR residues which are non-

10   homologous with the aligned consensus FR sequence, and for each such non-homologous import antibody FR residue, determining if the corresponding consensus antibody residue represents a residue which is highly conserved across all species at that site, and if it is so conserved, preparing a humanized antibody which comprises the consensus antibody amino acid residue at that site.

15   Certain alternate embodiments of the methods of this invention comprise obtaining the amino acid sequence of at least a portion of an import, non-human antibody variable domain having a CDR and a FR, obtaining the amino acid sequence of at least a portion of a consensus antibody variable domain having a CDR and a FR, substituting the non-human CDR for the human CDR in the consensus antibody variable domain, and then substituting an

20   amino acid residue for the consensus amino acid residue at at least one of the following sites:

a.   (in the FR of the variable domain of the light chain) 4L, 35L, 36L, 38L, 43L, 44L, 58L, 46L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71L, 73L, 85L, 87L, 98L, or

b.   (in the FR of the variable domain of the heavy chain) 2H, 4H, 24H, 36H, 37H,
25   39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H.

In preferred embodiments, the non-CDR residue substituted at the consensus FR site is the residue found at the corresponding location of the non-human antibody.

Optionally, this just-recited embodiment comprises the additional steps of following the
30   method steps appearing at the beginning of this summary and determining whether a particular amino acid residue can reasonably be expected to have undesirable effects.

This invention also relates to a humanized antibody comprising the CDR sequence of an import, non-human antibody and the FR sequence of a human antibody, wherein an amino acid residue within the human FR sequence located at any one of the sites 4L, 35L, 36L, 38L,
35   43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71L, 73L, 85L, 87L, 98L, 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H has been substituted by another residu . In preferred emb dim nts, the r sidue substituted at the human FR sit is the residue found at

-6-

the   rr sponding l cation of the n n-human antibody from which the non-human CDR was obtained. In other embodiments, no human FR residue other than th se set forth in this group has been substituted.

This invention also encompasses specific humanized antibody variable domains, and
5   isolated polypeptides having homology with the following sequences.

1. SEQ. ID NO. 1, which is the light chain variable domain of a humanized version of muMAb4D5:

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLESGVP SRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQTKVEIKRT

10   2. SEQ. ID NO. 2, which is the heavy chain variable domain of a humanized version of muMAb4D5):

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTR YADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDVWGQGTLV TVSS

15   In another aspect, this invention provides a consensus antibody variable domain amino acid sequence for use in the preparation of humanized antibodies, methods for obtaining, using, and storing a computer representation of such a consensus sequence, and computers comprising the sequence data of such a sequence. In one embodiment, the following consensus antibody variable domain amino acid sequences are provided:

20

SEQ. ID NO. 3 (light chain):

DIQMTQSPSSLSASVGDRVTITCRASQDVSSYLAWYQQKPGKAPKLLIYAASSLESGVP SRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSLPYTFGQGTKVEIKRT, and

25   SEQ. ID NO. 4 (heavy chain):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSWVRQAPGKGLEWVAVISENGGYT RYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDVWGQGTL VTVSS

30   **Brief Description of the Drawings**

FIGURE 1A shows the comparison of the V_L domain amino acid residues of muMAb4D5, huMAb4D5, and a consensus sequence (Fig. 1A, SEQ.ID NO. 5, SEQ. ID NO. 1 and SEQ. ID NO. 3, respectively). FIGURE 1B shows the comparison between the V_H domain amino acid residues of the muMAb4d5, huMAb4D5, and a consensus sequence (Fig.
35   1B, SEQ. ID NO. 6, SEQ. ID NO. 2 and SEQ. ID NO. 4, respectively). Both Figs 1A and 1B use the generally accepted numbering scheme from Kabat, E. A., et al., Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, MD (1987)). In both Fig. 1A and Fig. 1B, the CDR residu s determined according to a standard s quence definition (as

in Kabat, E. A. *et al., Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987)) are indicated by the first und rlining ben ath the sequ nces, and the CDR residues determined according to a structural definition (as in Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901-917 (1987)) are indicated by the second, lower underlines. The mismatches between genes are shown by the vertical lines.　　　FIGURE 2 shows a scheme for humanization of muMAb4D5 V_L and V_H by gene conversion mutagenesis.

　　　FIGURE 3 shows the inhibition of SK-BR-3 proliferation by MAb4D5 variants. Relative cell proliferation was determined as described (Hudziak, R. M. *et al., Molec. Cell. Biol.* 9:1165-1172 (1989)) and data (average of triplicate determinations) are presented as a percentage of results with untreated cultures for muMAb4D5 (I), huMAb4D5-8 (n) and huMAb4D5-1 (I).

　　　FIGURE 4 shows a stereo view of α-carbon tracing for a model of huMAb4D5-8 V_L and V_H . The CDR residues (Kabat, E. A. *et al., Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987)) are shown in bold and side chains of V_H residues A71, T73, A78, S93, Y102 and V_L residues Y55 plus R66 (see Table 3) are shown.

　　　FIGURE 5 shows an amino acid sequence comparison of V_L (top panel) and V_H (lower panel) domains of the murine anti-CD3 monoclonal Ab UCHT1 (muxCD3, Shalaby *et al., J. Exp. Med.* 175, 217-225 (1992) with a humanized variant of this antibody (huxCD3v9). Also shown are consensus sequences (most commonly occurring residue or pair of residues) of the most abundant human subgroups, namely V_L κ 1 and V_H III upon which the humanized sequences are based (Kabat, E. A. *et al., Sequences of Proteins of Immunological Interest*, 5th edition, National Institutes of Health, Bethesda, MD, USA (1991)). The light chain sequences-＿ -muxCD3, huxCD3v9 and huxI--correspond to SEQ.ID.NOs 16, 17, and 18, respectively. The heavy chain sequences--muxCD3, huxCD3v9 and huxI--correspond to SEQ.ID.NOs 19, 20, and 21, respectively. Residues which differ between muxCD3 and huxCD3v9 are identified by an asterisk (*), whereas those which differ between humanized and consensus sequences are identified by a sharp sign (#). A bullet (○) denotes that a residue at this position has been found to contact antigen in one or more crystallographic structures of antibody/antigen complexes (Kabat *et al.*, 1991; Mian, I. S. *et al., J. Mol. Biol.* 217, 133-151 (1991)). The location of CDR residues according to a sequence definition (Kabat *et al.*, 1991) and a structural definition (Chothia and Lesk, *supra* 1987) are shown by a line and carats (^) beneath the sequences, respectively.

　　　FIGURE 6A compares murine and humanized amino acid sequences for the heavy chain of an anti-CD18 antibody. H52H4-160 (SEQ. ID. NO. 22) is the murine sequence, and pH52-8.0 (SEQ. ID. NO. 23) is the humanized heavy chain sequence. pH52-8.0 residue 143S is the final amino acid in the variable heavy chain domain V_H, and residue 144A is the first amino acid in the constant heavy chain domain C_{H1}.

FIGURE 6B ompares murine and humanized amino acid s quences for the light chain

f an anti-CD18 antibody. H52L6-158 (SEQ. ID. NO. 24) is the murine sequence, and pH52-

9.0 (SEQ. ID. NO. 25) is the humanized light chain sequence. pH52-9.0 residue 128T is the

final amino acid in the light chain variable domain V_L, and residue 129V is the first amino acid

5   in the light chain constant domain C_L.

FIGURE 7A shows an amino acid sequence alignment of the sequences of the heavy

chains of thirteen humanized anti-CD18 (H52) variants (SEQ. ID. NOs 26-38).

FIGURE 7B shows an amino acid sequence alignment of two humanized anti-CD18

(H52) light chain variants (SEQ. ID. NOs 39-40).

10

Detailed Description of the Invention

Definitions

In general, the following words or phrases have the indicated definitions when used in

the description, examples, and claims:

15   The murine monoclonal antibody known as muMAb4D5 (Fendly, B. M. et al., Cancer

Res. 50:1550-1558 (1990)) is directed against the extracellular domain (ECD) of p185^{HER2}.

The muMAb4D5 and its uses are described in PCT application WO 89/06692 published 27

July 1989. This murine antibody was deposited with the ATCC and designated ATCC CRL

10463. In this description and claims, the terms muMAb4D5, chMAb4D5 and huMAb4D5

20   represent murine, chimerized and humanized versions of the monoclonal antibody 4D5,

respectively.

A humanized antibody for the purposes herein is an immunoglobulin amino acid

sequence variant or fragment thereof which is capable of binding to a predetermined antigen

and which comprises a FR region having substantially the amino acid sequence of a human

25   immunoglobulin and a CDR having substantially the amino acid sequence of a non-human

immunoglobulin.

Generally, a humanized antibody has one or more amino acid residues introduced into

it from a source which is non-human. These non-human amino acid residues are referred to

herein as "import" residues, which are typically taken from an "import" antibody domain,

30   particularly a variable domain. An import residue, sequence, or antibody has a desired affinity

and/or specificity, or other desirable antibody biological activity as discussed herein.

In general, the humanized antibody will comprise substantially all of at least one, and

typically two, variable domains (Fab, Fab', F(ab')$_2$, Fabc, Fv) in which all or substantially all

of the CDR regions correspond to those of a non-human immunoglobulin and all or

35   substantially all of the FR regions are those of a human immunoglobulin consensus sequence.

The humanized antibody optimally also will comprise at least a portion of an immunoglobulin

constant regi n (Fc), typically that of a human immunoglobulin. Ordinarily, the antibody will

c ntain both the light chain as well as at least the variable domain of a heavy chain. The antibody also may include the CH1, hinge, CH2, CH3, and CH4 regions f the heavy chain.

The humanized antib dy will be selected fr m any class of immunoglobulins, including IgM, IgG, IgD, IgA and IgE, and any isotype, including IgG1, IgG2, IgG3 and IgG4. Usually the constant domain is a complement fixing constant domain where it is desired that the humanized antibody exhibit cytotoxic activity, and the class is typically IgG_1. Where such cytotoxic activity is not desirable, the constant domain may be of the IgG_2 class. The humanized antibody may comprise sequences from more than one class or isotype, and selecting particular constant domains to optimize desired effector functions is within the ordinary skill in the art.

The FR and CDR regions of the humanized antibody need not correspond precisely to the parental sequences, e.g., the import CDR or the consensus FR may be mutagenized by substitution, insertion or deletion of at least one residue so that the CDR or FR residue at that site does not correspond to either the consensus or the import antibody. Such mutations, however, will not be extensive. Usually, at least 75% of the humanized antibody residues will correspond to those of the parental FR and CDR sequences, more often 90%, and most preferably greater than 95%.

In general, humanized antibodies prepared by the method of this invention are produced by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen.

Residues that influence antigen binding are defined to be residues that are substantially responsible for the antigen affinity or antigen specificity of a candidate immunoglobulin, in a positive or a negative sense. The invention is directed to the selection and combination of FR residues from the consensus and import sequence so that the desired immunoglobulin characteristic is achieved. Such desired characteristics include increases in affinity and greater specificity for the target antigen, although it is conceivable that in some circumstances the opposite effects might be desired. In general, the CDR residues are directly and most substantially involved in influencing antigen binding (although not all CDR residues are so involved and therefore need not be substituted into the consensus sequence). However, FR residues also have a significant effect and can exert their influence in at least three ways: They may noncovalently directly bind to antigen, they may interact with CDR residues and they may affect the interface between the heavy and light chains.

A residu that noncovalently directly binds t antigen is one that, by three dimensional analysis, is reas nably expect d t n ncovalently directly bind to antigen. Typically, it is necessary to impute the position of antigen from the spatial location of neighboring CDRs and the dimensions and structure of the target antigen. In general, only those humanized antibody residues that are capable of forming salt bridges, hydrogen bonds, or hydrophobic interactions are likely to be involved in non-covalent antigen binding, however residues which have atoms which are separated from antigen spatially by 3.2 Angstroms or less may also non-covalently interact with antigen. Such residues typically are the relatively larger amino acids having the side chains with the greatest bulk, such as tyrosine, arginine, and lysine. Antigen-binding FR residues also typically will have side chains that are oriented into an envelope surrounding the solvent oriented face of a CDR which extends about 7 Angstroms into the solvent from the CDR domain and about 7 Angstroms on either side of the CDR domain, again as visualized by three dimensional modeling.

A residue that interacts with a CDR generally is a residue that either affects the conformation of the CDR polypeptide backbone or forms a noncovalent bond with a CDR residue side chain. Conformation-affecting residues ordinarily are those that change the spatial position of any CDR backbone atom (N, $C\alpha$, C, O, $C\beta$) by more than about 0.2 Angstroms. Backbone atoms of CDR sequences are displaced for example by residues that interrupt or modify organized structures such as beta sheets, helices or loops. Residues that can exert a profound affect on the conformation of neighboring sequences include proline and glycine, both of which are capable of introducing bends into the backbone. Other residues that can displace backbone atoms are those that are capable of participating in salt bridges and hydrogen bonds.

A residue that interacts with a CDR side chain is one that is reasonably expected to form a noncovalent bond with a CDR side chain, generally either a salt bridge or hydrogen bond. Such residues are identified by three dimensional positioning of their side chains. A salt or ion bridge could be expected to form between two side chains positioned within about 2.5 - 3.2 Angstroms of one another that bear opposite charges, for example a lysinyl and a glutamyl pairing. A hydrogen bond could be expected to form between the side chains of residue pairs such as seryl or threonyl with aspartyl or glutamyl (or other hydrogen accepting residues). Such pairings are well known in the protein chemistry art and will be apparent to the artisan upon three dimensional modeling of the candidate immunoglobulin.

Immunoglobulin residues that affect the interface between heavy and light chain variable regions ("the V_L - V_H interface") are those that affect the proximity or orientation of the two chains with respect to one another. Certain residues involved in interchain interactions are already known and include V_L residues 34, 36, 38, 44, 46, 87, 89, 91, 96, and 98 and V_H residues 35, 37, 39, 45, 47, 91, 93, 95, 100, and 103 (utilizing the nomenclature set forth in Kabat *et al.*, *Sequences of Proteins of Immunological Interest*

(National Institutes of Health, B thesda, MD, 1987)). Additional residues are newly identified by the invent rs herein, and include 43L, 85L, 43H and 60H. While thes residues are indicated for IgG only, they are applicable across species. In the practice of this invention, import antibody residues that are reasonably expected to be involved in interchain interactions

5   are selected for substitution into the consensus sequence. It is believed that heretofore no humanized antibody has been prepared with an intrachain-affecting residue selected from an import antibody sequence.

Since it is not entirely possible to predict in advance what the exact impact of a given substitution will be it may be necessary to make the substitution and assay the candidate

10   antibody for the desired characteristic. These steps, however, are *per se* routine and well within the ordinary skill of the art.

CDR and FR residues are determined according to a standard sequence definition (Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, National Institutes of Health, Bethesda MD (1987), and a structural definition (as in Chothia and Lesk, *J. Mol. Biol.* 196:901-917

15   (1987). Where these two methods result in slightly different identifications of a CDR, the structural definition is preferred, but the residues identified by the sequence definition method are considered important FR residues for determination of which framework residues to import into a consensus sequence.

Throughout this description, reference is made to the numbering scheme from Kabat,

20   E. A., *et al.*, *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD (1987) and (1991). In these compendiums, Kabat lists many amino acid sequences for antibodies for each subclass, and lists the most commonly occurring amino acid for each residue position in that subclass. Kabat uses a method for assigning a residue number to each amino acid in a listed sequence, and this method for assigning residue

25   numbers has become standard in the field. The Kabat numbering scheme is followed in this description. For purposes of this invention, to assign residue numbers to a candidate antibody amino acid sequence which is not included in the Kabat compendium, one follows the following steps. Generally, the candidate sequence is aligned with any immunoglobulin sequence or any consensus sequence in Kabat. Alignment may be done by hand, or by

30   computer using commonly accepted computer programs; an example of such a program is the Align 2 program discussed in this description. Alignment may be facilitated by using some amino acid residues which are common to most Fab sequences. For example, the light and heavy chains each typically have two cysteines which have the same residue numbers; in V_L domain the two cysteines are typically at residue numbers 23 and 88, and in the V_H domain

35   the two cysteine residues are typically numbered 22 and 92. Framework residues generally, but not always, have approximately the same number of residues, however the CDRs will vary in size. For example, in the case of a CDR from a candidate sequence which is longer than the CDR in the sequence in Kabat to which it is aligned, typically suffixes are add d t the

residue number t indicate the insertion of additional residues (see, e.g. r sidues 100abcde in Fig. 5). For candidate sequ nces which, f r example, align with a Kabat sequence f r residues 34 and 36 but hav no residue between them to align with residue 35, th number 35 is simply not assigned to a residue.

Thus, in humanization of an import variable sequence, where one cuts out an entire human or consensus CDR and replaces it with an import CDR sequence, (a) the exact number of residues may be swapped, leaving the numbering the same, (b) fewer import amino acid residues may be introduced than are cut, in which case there will be a gap in the residue numbers, or (c) a larger number of amino acid residues may be introduced then were cut, in which case the numbering will involve the use of suffixes such as 100abcde.

The terms "consensus sequence" and "consensus antibody" as used herein refers to an amino acid sequence which comprises the most frequently occurring amino acid residues at each location in all immunoglobulins of any particular subclass or subunit structure. The consensus sequence may be based on immunoglobulins of a particular species or of many species. A "consensus" sequence, structure, or antibody is understood to encompass a consensus human sequence as described in certain embodiments of this invention, and to refer to an amino acid sequence which comprises the most frequently occurring amino acid residues at each location in all human immunoglobulins of any particular subclass or subunit structure. This invention provides consensus human structures and consensus structures which consider other species in addition to human.

The subunit structures of the five immunoglobulin classes in humans are as follows:

Class	Heavy Chain	Subclasses	Light Chain	Molecular Formula
IgG	γ	$\gamma1, \gamma2, \gamma3, \gamma4$	κ or λ	$(\gamma_2\kappa_2)$, $(\gamma_2\lambda_2)$
IgA	α	$\alpha1, \alpha2$	κ or λ	$(\alpha_2\kappa_2)_n^*$, $(\alpha_2\lambda_2)_n^*$
IgM	μ	none	κ or λ	$(\mu_2\kappa_2)_5$, $(\mu_2\lambda_2)_5$
IgD	δ	none	κ or λ	$(\delta_2\kappa_2)$, $(\delta_2\lambda_2)$
IgE	ϵ	none	κ or λ	$(\epsilon_2\kappa_2)$, $(\epsilon_2\lambda_2)$

($^*_n$ may equal 1, 2, or 3)

In preferred embodiments of an IgGγ1 human consensus sequence, the consensus variable domain sequences are derived from the most abundant subclasses in the sequence compilation of Kabat et al., *Sequences of Proteins of Immunological Interest*, National Institutes of Health, Bethesda MD (1987); namely V_L κ subgroup I and V_H group III. In such preferred embodiments, the V_L consensus domain has the amino acid sequence: DIQMTQSPSSLSASVGDRVTITCRASQDVSSYLAWYQQKPGKAPKLLIYAASSLESGVPSRFSGSGS GTDFTLTISSLQPEDFATYYCQQYNSLPYTFGQGTKVEIKRT (SEQ. ID NO. 3); the V_H consensus domain has the amino acid sequence: EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSWVRQAPGKGLEWVAVISENGGYTRYADSVK GRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDVWGQGTLVTVSS(SEQ. ID NO. 4).

These sequences include consensus CDRs as well as consensus FR residues (see for example in Fig. 1).

While not wishing t be limited to any particular theories, it may be that these preferred embodiments are less likely to be immunogenic in an individual than less abundant ubclasses.

5   However, in other embodiments, the consensus sequence is derived from other subclasses of human immunoglobulin variable domains. In yet other embodiments, the consensus sequence is derived from human constant domains.

. Identity or homology with respect to a specified amino acid sequence of this invention is defined herein as the percentage of amino acid residues in a candidate sequence that are identical

10   with the specified residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. None of N-terminal, C-terminal or internal extensions, deletions, or insertions into the specified sequence shall be construed as affecting homology. All sequence alignments called for in this invention are such maximal homology alignments. While such

15   alignments may be done by hand using conventional methods, a suitable computer program is the "Align 2" program for which protection is being sought from the U.S. Register of Copyrights (Align 2, by Genentech, Inc., application filed 9 December 1991).

"Non-homologous" import antibody residues are those residues which are not identical to the amino acid residue at the analogous or corresponding location in a consensus sequence, after

20   the import and consensus sequences are aligned.

The term "computer representation" refers to information which is in a form that can be manipulated by a computer. The act of storing a computer representation refers to the act of placing the information in a form suitable for manipulation by a computer.

This invention is also directed to novel polypeptides, and in certain aspects, isolated novel

25   humanized anti-p185^{HER2} antibodies are provided. These novel anti-p185^{HER2} antibodies are sometimes collectively referred to herein as huMAb4D5, and also sometimes as the light or heavy chain variable domains of huMAb4D5, and are defined herein to be any polypeptide sequence which possesses a biological property of a polypeptide comprising the following polypeptide sequence:

30   DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLESGVPSRFS
          GSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRT(SEQ. ID NO. 1, which is
          the light chain variable domain of huMAb4D5); or
          EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYAD
          SVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDVWGQGTLVTVSSEQ.

35   ID NO. 2, which is the heavy chain variable domain of huMAb4D5).

"Biological property", as relates for example to anti-p185^{HER2}, for the purposes herein means an *in vivo* effector or antigen-binding function or activity that is directly or indirectly performed by huMAb4D5 (whether in its native or denatured conformation). Effector functions include p185^{HER2} binding, any hormonal or hormonal antagonist activity, any mitogenic or agonist

40   or antagonist activity, any cytotoxic activity. An antigenic function means possession of an

epitope r antigenic site that is capable f cross-reacting with antibodies raised against the polypeptide sequence f huMAb4D5.

Biologically active huMAb4D5 is defined herein as a polypeptide that shares an effector function of huMAb4D5. A principal known effector function of huMAb4D5 is its ability to bind to
5   p185^{HER2}.

Thus, the biologically active and antigenically active huMAb4D5 polypeptides that are the subject of certain embodiments of this invention include the sequence of the entire translated nucleotide sequence of huMAb4D5; mature huMAb4D5; fragments thereof having a consecutive sequence of at least 5, 10, 15, 20, 25, 30 or 40 amino acid residues comprising sequences from
10   muMAb4D5 plus residues from the human FR of huMAb4D5; amino acid sequence variants of huMAb4D5 wherein an amino acid residue has been inserted N- or C-terminal to, or within, huMAb4D5 or its fragment as defined above; amino acid sequence variants of huMAb4D5 or its fragment as defined above wherein an amino acid residue of huMAb4D5 or its fragment as defined above has been substituted by another residue, including predetermined mutations by, e.g., site-
15   directed or PCR mutagenesis; derivatives of huMAb4D5 or its fragments as defined above wherein huMAb4D5 or its fragments have been covalent modified, by substitution, chemical, enzymatic, or other appropriate means, with a moiety other than a naturally occurring amino acid; and glycosylation variants of huMAb4D5 (insertion of a glycosylation site or deletion of any glycosylation site by deletion, insertion or substitution of suitable residues). Such fragments and
20   variants exclude any polypeptide heretofore identified, including muMAb4D5 or any known polypeptide fragment, which are anticipatory order 35 U.S.C.102 as well as polypeptides obvious thereover under 35 U.S.C. 103.

An "isolated" polypeptide means polypeptide which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural
25   environment are materials which would interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, for example, a polypeptide product comprising huMAb4D5 will be purified from a cell culture or other synthetic environment (1) to greater than 95% by weight of protein as determined by the Lowry method, and most preferably more than 99% by weight,
30   (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a gas- or liquid-phase sequenator (such as a commercially available Applied Biosystems sequenator Model 470, 477, or 473), or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated huMAb4D5 includes huMAb4D5 in situ within recombinant cells since at least one component of
35   the huMAb4D5 natural environment will not be present. Ordinarily, however, isolated huMAb4D5 will be prepared by at least one purification step.

In accordance with this invention, huMAb4D5 nucleic acid is RNA or DNA containing greater than ten bases that encodes a biologically or antigenically active huMAb4D5, is complementary t nucleic acid sequence enc ding such huMAb4D5, or hybridizes t nucl ic acid sequence

encoding such huMAb4D5 and remains stably bound to it under stringent c nditions, and comprises nucleic acid from a muMAb4D5 CDR and a human FR region.

Preferably, the huMAb4D5 nucleic acid encodes a polypeptide sharing at least 75% sequence identity, more preferably at least 80%, still more preferably at least 85%, even more
5   preferably at 90%, and most preferably 95%, with the huMAb4D5 amino acid sequence. Preferably, a nucleic acid molecule that hybridizes to the huMAb4D5 nucleic acid contains at least 20, more preferably 40, and most preferably 90 bases. Such hybridizing or complementary nucleic acid, however, is further defined as being novel under 35 U.S.C. 102 and unobvious under 35 U.S.C. 103 over any prior art nucleic acid.

10   Stringent conditions are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0/1% NaDodSO$_4$ at 50° C; (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0/1% Ficoll/0/1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42° C; or (3) employ
15   50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 g/ml), 0.1% SDS, and 10% dextran sulfate at 42 C, with washes at 42 C in 0.2 x SSC and 0.1% SDS.

The term "control sequences" refers to DNA sequences necessary for the expression of an
20   operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another
25   nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA
30   sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

An "exogenous" element is defined herein to mean nucleic acid sequence that is foreign to
35   the cell, or homologous to the cell but in a position within the host cell nucleic acid in which the element is ordinarily not found.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard
40   for the number f transfers. It is also understood that all pr geny may not be precisely identical

-16-

in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

"Oligonucleotides" are short-length, single- or double-stranded polydeoxynucleotides that
5   are chemically synthesized by known methods (such as phosphotriester, phosphite, or phosphoramidite chemistry, using solid phase techniques such as described in EP 266,032 published 4 May 1988, or via deoxynucleoside H-phosphonate intermediates as described by Froehler et al., Nucl. Acids Res., 14: 5399-5407[1986]). They are then purified on polyacrylamide gels.

10   The technique of "polymerase chain reaction," or "PCR," as used herein generally refers to a procedure wherein minute amounts of a specific piece of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Pat. No. 4,683,195 issued 28 July 1987. Generally, sequence information from the ends of the region of interest or beyond needs to be available, such that oligonucleotide primers can be designed; these primers will be identical or similar in sequence to
15   opposite strands of the template to be amplified. The 5' terminal nucleotides of the two primers may coincide with the ends of the amplified material. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, etc. See generally Mullis et al., Cold Spring Harbor Symp. Quant. Biol., 51: 263 (1987); Erlich, ed., PCR Technology, (Stockton Press, NY,
20   1989). As used herein, PCR is considered to be one, but not the only, example of a nucleic acid polymerase reaction method for amplifying a nucleic acid test sample, comprising the use of a known nucleic acid (DNA or RNA) as a primer and utilizes a nucleic acid polymerase to amplify or generate a specific piece of nucleic acid or to amplify or generate a specific piece of nucleic acid which is complementary to a particular nucleic acid.

25

Suitable Methods for Practicing the Invention

Some aspects of this invention include obtaining an import, non-human antibody variable domain, producing a desired humanized antibody sequence and for humanizing an antibody gene sequence are described below. A particularly preferred method of changing a gene sequence, such
30   as gene conversion from a non-human or consensus sequence into a humanized nucleic acid sequence, is the cassette mutagenesis procedure described in Example 1. Additionally, methods are given for obtaining and producing antibodies generally, which apply equally to native non-human antibodies as well as to humanized antibodies.

Generally, the antibodies and antibody variable domains of this invention are conventionally
35   prepared in recombinant cell culture, as described in more detail below. Recombinant synthesis is preferred for reasons of safety and economy, but it is known to prepare peptides by chemical synthesis and to purify them from natural sources; such preparations are included within the definition of antibodies herein.

40

Molecular Modeling

        An integral step in our approach to antibody humanization is construction of computer graphics models of the import and humanized antibodies. These models are used to determine if the six complementarity-determining regions (CDRs) can be successfully transplanted from the

5   import framework to a human one and to determine which framework residues from the import antibody, if any, need to be incorporated into the humanized antibody in order to maintain CDR conformation. In addition, analysis of the sequences of the import and humanized antibodies and reference to the models can help to discern which framework residues are unusual and thereby might be involved in antigen binding or maintenance of proper antibody structure.

10   All of the humanized antibody models of this invention are based on a single three-dimensional computer graphics structure hereafter referred to as the consensus structure. This consensus structure is a key distinction from the approach of previous workers in the field, who typically begin by selecting a human antibody structure which has an amino acid sequence which is similar to the sequence of their import antibody.

15   The consensus structure of one embodiment of this invention was built in five steps as described below.

        Step 1:   Seven Fab X-ray crystal structures from the Brookhaven Protein Data Bank were used (entries 2FB4, 2RHE, 3FAB, and 1REI which are human structures, and 2MCP, 1FBJ, and 2HFL which are murine structures). For each structure, protein mainchain geometry and

20   hydrogen bonding patterns were used to assign each residue to one of three secondary structure types: alpha-helix, beta-strand or other (i.e. non-helix and non-strand). The immunoglobulin residues used in superpositioning and those included in the consensus structure are shown in Table 1.

Table I

25   Immunoglobulin Residues Used In Superpositioning and Those Included in the

Consensus Structure

$V_L K$ domain

Ig[a]	2FB4	2RHE	2MCP	3FAB	1FBJ	2HFL	1REI	Consensus[b]
								2-11
	18-24	18-24	19-25	18-24	19-25	19-25	19-25	16-27
	32-37	34-39	39-44	32-37	32-37	32-37	33-38	33-39
								41-49
	60-66	62-68	67-72	53-66	60-65	60-65	61-66	59-77
	69-74	71-76	76-81	69-74	69-74	69-74	70-75	
	84-88	86-90	91-95	84-88	84-88	84-88	85-89	82-91
								101-105
RMS[c]		0.40	0.60	0.53	0.54	0.48	0.50	

-18-

Table I (Con't)

V$_H$ domain

Ig[a]	2FB4	2MCP	3FAB	1FBJ	2HFL	Consensus[b]
						3-8
	18-25	18-25	18-25	18-26	18-25	17-23
	34-39	34-39	34-39	34-39	34-39	33-41
	46-52	46-52	46-52	46-52	46-52	45-51
	57-61	59-63	56-60	57-61	57-61	57-61
	68-71	70-73	67-70	68-71	68-71	66-71
	78-84	80-86	77-83	78-84	78-84	75-82
	92-99	94-101	91-98	92-99	92-99	88-94
						102-108
RMS[c]		0.43	0.85	0.62	0.91	
RMS[d]	0.91	0.73	0.77	0.92		

a   Four-letter code for Protein Data Bank file.
b   Residue numbers for the crystal structures are taken from the Protein Data Bank files. Residue numbers for the consensus structure are according to Kabat et al.
c.   Root-mean-square deviation in Å for (N.Cα,C) atoms superimposed on 2FB4.
d.   Root-mean-square deviation in Å for (N,Cα,C) atoms superimposed on 2HFL.

Step 2:   Having identified the alpha-helices and beta-strands in each of the seven structures, the structures were superimposed on one another using the INSIGHT computer program (Biosym Technologies, San Diego, CA) as follows: The 2FB4 structure was arbitrarily chosen as the template (or reference) structure. The 2FB4 was held fixed in space and the other six structures rotated and translated in space so that their common secondary structural elements (i.e. alpha-helices and beta-strands) were oriented such that these common elements were as close in position to one another as possible. (This superpositioning was performed using accepted mathematical formulae rather than actually physically moving the structures by hand.)

Step 3:   With the seven structures thus superimposed, for each residue in the template (2FB4) Fab one calculates the distance from the template alpha-carbon atom (Cα) to the analogous Cα atom in each of the other six superimposed structures. This results in a table of Cα-Cα distances for each residue position in the sequence. Such a table is necessary in order to determine which residue positions will be included in the consensus model. Generally, if all Cα-Cα distances for a given residue position were $\leq$ 1.0Å, that position was included in the consensus structure. If for a given position only one Fab crystal structure was > 1.0Å, the position was included but the outlying crystal structure was not included in the next step (for this position only). In general, the seven β-strands were included in the consensus structure while some of the loops connecting the β-strands, e.g. complementarity-determining regions (CDRs), were not included in view of Cα divergence.

Step 4:   For each residue which was included in the consensus structure after step 3, the average of the coordinates for individual mainchain N, Cα, C, O and Cβ atoms were calculated. Due to the averaging procedure, as well as variation in bond length, bond angle and dihedral angle among the crystal structures, this "average" structure contained some bond lengths and angles which deviated from standard geometry. For purposes of this invention, "standard geometry" is understood to include geometries commonly accepted as typical, such as the compilation of bond lengths and angles from small molecule structures in Weiner, S.J. *et. al., J. Amer. Chem. Soc.,* 106: 765-784 (1984).

Step 5: .   In order to correct these deviations, the final step was to subject the "average" structure to 50 cycles of energy minimization (DISCOVER program, Biosym Technologies) using the AMBER (Weiner, S.J. *et. al., J. Amer. Chem. Soc.,* 106: 765-784 (1984)) parameter set with only the Cα coordinates fixed (i.e. all other atoms are allowed to move) (energy minimization is described below). This allowed any deviant bond lengths and angles to assume a standard (chemically acceptable) geometry. See Table II.

Table II

Average Bond Lengths and Angles for "Average" (Before) and

Energy-Minimized Consensus (After 50 Cycles) Structures .

	V_LK before (Å)	V_LK after (Å)	V_M before (Å)	V_M after (Å)	Standard Geometry (Å)
N-Cα	1.459(0.012)	1.451(0.004)	1.451(0.023)	1.452(0.004)	1.449
Cα-C	1.515(0.012)	1.523(0.005)	1.507(0.033)	1.542(0.005)	1.522
O=C	1.208(0.062)	1.229(0.003)	1.160(0.177)	1.231(0.003)	1.229
C-N	1.288(0.049)	1.337(0.002)	1.282(0.065)	1.335(0.004)	1.335
Cα-Cβ	1.508(0.026)	1.530(0.002)	1.499(0.039)	1.530(0.002)	1.526
	(°)	(°)	(°)	(°)	(°)
C-N-Cα	123.5(4.2)	123.8(1.1)	125.3(4.6)	124.0(1.1)	121.9
N-Cα-C	110.0(4.0)	109.5(1.9)	110.3(2.8)	109.5(1.6)	110.1
Cα-C-N	116.6(4.0)	116.6(1.2)	117.6(5.2)	116.6(0.8)	116.6
O=C-N	123.1(4.1)	123.4(0.6)	122.2(4.9)	123.3(0.4)	122.9
N-Cα-Cβ	110.3(2.1)	109.8(0.7)	110.6(2.5)	109.8(0.6)	109.5
Cβ-Cα-C	111.4(2.4)	111.1(0.7)	111.2(2.2)	111.1(0.6)(	111.1

Values in parentheses are standard deviations. Note that while some bond length and angle averages did not change appreciably after energy-minimization, the corresponding standard deviations are reduced due to deviant geometries assuming standard values after energy-minimization. Standard geometry values are from the AMBER forcefield as implemented in DISCOVER (Biosym Technologies).

The c nsensus structure might conceivably be d pendent upon which crystal structure was chosen as the template on which the others were superimp sed. As a test, the entir procedure was repeated using the crystal structure with the worst superposition versus 2FB4, i.e. the 2HFL Fab structure, as the new template (reference). The two consensus structures compare favorably (root-mean-squared deviation of 0.11Å for all N, Cα and C atoms).

Note that the consensus structure only includes mainchain (N, Cα, C, O, Cβ atoms) coordinates for only those residues which are part of a conformation *common* to all seven X-ray crystal structures. For the Fab structures, these include the common β-strands (which comprise two β-sheets) and a few non-CDR loops which connect these β-strands. The consensus structure does *not* include CDRs or sidechains, both of which vary in their conformation among the seven structures. Also, note that the consensus structure includes only the VL and VH domains.

This consensus structure is used as the archetype. It is not particular to any species, and has only the basic shape without side chains. Starting with this consensus structure the model of *any* import, human, or humanized Fab can be constructed as follows. Using the amino acid sequence of the particular antibody VL and VH domains of interest, a computer graphics program (such as INSIGHT, Biosym Technologies) is used to add sidechains and CDRs to the consensus structure. When a sidechain is added, its conformation is chosen on the basis of known Fab crystal structures (see the Background section for publications of such crystal structures) and rotamer libraries (Ponder, J.W. & Richards, F. M., *J. Mol. Biol.* 193: 775-791 (1987)). The model also is constructed so that the atoms of the sidechain are positioned so as to not collide with other atoms in the Fab.

CDRs are added to the model (now having the backbone plus side chains) as follows. The size (i.e. number of amino acids) of each import CDR is compared to canonical CDR structures tabulated by Chothia *et al.*, *Nature*, 342:877-883 (1989)) and which were derived from Fab crystals. Each CDR sequence is also reviewed for the presence or absence of certain specific amino acid residues which are identified by Chothia as structurally important: e.g. light chain residues 29 (CDR1) and 95 (CDR3), and heavy chain residues 26, 27, 29 (CDR1) and 55 (CDR2). For light chain CDR2, and heavy chain CDR3, only the size of the CDR is compared to the Chothia canonical structure. If the size and sequence (i.e. inclusion of the specific, structurally important residues as denoted by Chothia *et al.*) of the import CDR agrees in size and has the same structurally important residues as those of a canonical CDR, then the mainchain conformation of the import CDR in the model is taken to be the same as that of the canonical CDR. This means that the import sequence is assigned the structural configuration of the canonical CDR, which is then incorporated in the evolving model.

However, if no matching canonical CDR can be assigned for the import CDR, then one of two options can be exercised. First, using a program such as INSIGHT (Biosym Technologies), the Brookhaven Protein Data Bank can be searched for loops with a similar size

to that of th imp rt CDR and th se loops can be valuated as possible conformati ns for the import CDR in the m del. Minimally, such loops must xhibit a conformation in which no loop atom overlaps with other protein atoms. Second, one can use available programs which calculate possible loop conformations, assuming a given loop size, using methods such as
5   described by Bruccoleri *et al., Nature* 335: 564-568 (1988).

When all CDRs and sidechains have been added to the consensus structure to give the final model (import, human or humanized), the model is preferably subjected to energy minimization using programs which are available commercially (e.g. DISCOVER, Biosym Technologies). This technique uses complex mathematical formulae to refine the model by
10   performing such tasks as checking that all atoms are within appropriate distances from one another and checking that bond lengths and angles are within chemically acceptable limits.

Models of a humanized, import or human antibody sequence are used in the practice of this invention to understand the impact of selected amino acid residues of the activity of the sequence being modeled. For example, such a model can show residues which may be
15   important in antigen binding, or for maintaining the conformation of the antibody, as discussed in more detail below. Modeling can also be used to explore the potential impact of changing any amino acid residue in the antibody sequence.

Methods for Obtaining a Humanized Antibody Sequence

20   In the practice of this invention, the first step in humanizing an import antibody is deriving a consensus amino acid sequence into which to incorporate the import sequences. Next a model is generated for these sequences using the methods described above. In certain embodiments of this invention, the consensus human sequences are derived from the most abundant subclasses in the sequence compilation of Kabat et al. (Kabat, E. A. *et al.,*
25· *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987)), namely V_L κ subgroup I and V_H group III, and have the sequences indicated in the definitions above.

While these steps may be taken in different order, typically a structure for the candidate humanized antibody is created by transferring the at least one CDR from the non-human,
30   import sequence into the consensus human structure, after the entire corresponding human CDR has been removed. The humanized antibody may contain human replacements of the non-human import residues at positions within CDRs as defined by sequence variability (Kabat, E. A. *et al., Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987)) or as defined by structural variability (Chothia, C. & Lesk, A. M., *J.*
35   *Mol. Biol.* 196:901-917 (1987)). For example, huMAb4D5 contains human replacements of the muMAb4D5 residues at three positions within CDRs as defined by sequence variability (Kabat, E. A. *et al., Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987)) but not as defined by structural variability (Chothia, C. & Lesk,

-22-

A. M., *J. Mol. Biol.* 196:901-917 (1987)): V_L-CDR1 K24R, V_L-CDR2 R54L and V_L-CDR2 T56S.

Differences between the non-human import and the human consensus framework residues are individually investigated to determine their possible influence on CDR conformation and/or binding to antigen. Investigation of such possible influences is desirably performed through modeling, by examination of the characteristics of the amino acids at particular locations, or determined experimentally through evaluating the effects of substitution or mutagenesis of particular amino acids.

In certain preferred embodiments of this invention, a humanized antibody is made comprising amino acid sequence of an import, non-human antibody and a human antibody, utilizing the steps of:

    a.   obtaining the amino acid sequences of at least a portion of an import antibody variable domain and of a consensus human variable domain;

    b.   identifying Complementarity Determining Region (CDR) amino acid sequences in the import and the human variable domain sequences;

    c.   substituting an import CDR amino acid sequence for the corresponding human CDR amino acid sequence;

    d.   aligning the amino acid sequences of a Framework Region (FR) of the import antibody and the corresponding FR of the consensus antibody;

    e.   identifying import antibody FR residues in the aligned FR sequences that are non-homologous to the corresponding consensus antibody residues;

    f.   determining if the non-homologous import amino acid residue is reasonably expected to have at least one of the following effects:

        1.   non-covalently binds antigen directly,

        2.   interacts with a CDR; or

        3.   participates in the V_L - V_H interface; and

    g.   for any non-homologous import antibody amino acid residue which is reasonably expected to have at least one of these effects, substituting that residue for the corresponding amino acid residue in the consensus antibody FR sequence.

Optionally, one determines if any non-homologous residues identified in step (e) are exposed on the surface of the domain or buried within it, and if the residue is exposed but has none of the effects identified in step (f), one may retain the consensus residue.

Additionally, in certain embodiments the corresponding consensus antibody residues identified in step (e) above are selected from the group consisting of 4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71L, 73L, 85L, 87L, 98L, 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H (utilizing the numbering system set forth in Kabat, E.

A. *et al., Sequences of Proteins of Immunological Interest* (Nati nal Institutes of Health, Bethesda, MD, 1987)).

In preferred embodiments, the method of this invention comprises the additional steps of searching either or both of the import, non-human and the consensus variable domain
5   sequences for glycosylation sites, determining if the glycosylation is reasonably expected to be important for the desired antigen binding and biological activity of the antibody (i.e., determining if the glycosylation site binds to antigen or changes a side chain of an amino acid residue that binds to antigen, or if the glycosylation enhances or weakens antigen binding, or is important for maintaining antibody affinity). If the import sequence bears the glycosylation
10   site, it is preferred to substitute that site for the corresponding residues in the consensus human sequence if the glycosylation site is reasonably expected to be important. If only the consensus sequence, and not the import, bears the glycosylation site, it is preferred to eliminate that glycosylation site or substitute therefor the corresponding amino acid residues from the import sequence.

15   Another preferred embodiment of the methods of this invention comprises aligning import antibody and the consensus antibody FR sequences, identifying import antibody FR residues which are non-homologous with the aligned consensus FR sequence, and for each such non-homologous import antibody FR residue, determining if the corresponding consensus antibody residue represents a residue which is highly conserved across all species at that site,
20   and if it is so conserved, preparing a humanized antibody which comprises the consensus antibody amino acid residue at that site.

In certain alternate embodiments, one need not utilize the modeling and evaluation steps described above, and may instead proceed with the steps of obtaining the amino acid sequence of at least a portion of an import, non-human antibody variable domain having a
25   CDR and a FR, obtaining the amino acid sequence of at least a portion of a consensus human antibody variable domain having a CDR and a FR, substituting the non-human CDR for the human CDR in the consensus human antibody variable domain, and then substituting an amino acid residue for the consensus amino acid residue at at least one of the following sites:

a.   (in the FR of the variable domain of the light chain) 4L, 35L, 36L, 38L, 43L,
30   44L, 58L, 46L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71L, 73L, 85L,
                87L, 98L, or

b.   (in the FR of the variable domain of the heavy chain) 2H, 4H, 24H, 36H, 37H,
                39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H,
                78H, 91H, 92H, 93H, and 103H.

35   Preferably, the non-CDR residue substituted at the consensus FR site is the residue found at the corresponding location of the non-human antibody. If desired, one may utilize the other method steps described above for determining whether a particular amino acid residue can reasonably be expected to have undesirable effects, and remedying those effects.

If after making a humanized antibody according to the steps above and t sting its
activity one is not satisfied with the humanized antibody, one pref rably reexamines the
potential effects of the amino acids at the specific locations recited above. Additionally, it is
desirable to reinvestigate any buried residues which are reasonably expected to affect the V_L -
5   V_H interface but may not directly affect CDR conformation. It is also desirable to reevaluate
the humanized antibody utilizing the steps of the methods claimed herein.

In certain embodiments of this invention, amino acid residues in the consensus human
sequence are substituted for by other amino acid residues. In preferred embodiments,
residues from a particular non-human import sequence are substituted, however there are
10   circumstances where it is desired to evaluate the effects of other amino acids. For example,
If after making a humanized antibody according to the steps above and testing its activity one
is not satisfied with the humanized antibody, one may compare the sequences of other classes
or subgroups of human antibodies, or classes or subgroups of antibodies from the particular
non-human species, and determine which other amino acid side chains and amino acid
15   residues are found at particular locations and substituting such other residues.

Antibodies

Certain aspects of this invention are directed to natural antibodies and to monoclonal
antibodies, as illustrated in the Examples below and by antibody hybridomas deposited with
20.   the ATCC (as described below). Thus, the references throughout this description to the use
of monoclonal antibodies are intended to include the use of natural or native antibodies as well
as humanized and chimeric antibodies. As used herein, the term "antibody" includes the
antibody variable domain and other separable antibody domains unless specifically excluded.

In accordance with certain aspects of this invention, antibodies to be humanized (import
25·   antibodies) are isolated from continuous hybrid cell lines formed by the fusion of
antigen-primed immune lymphocytes with myeloma cells.

In certain embodiments, the antibodies of this invention are obtained by routine
screening. Polyclonal antibodies to an antigen generally are raised in animals by multiple
subcutaneous (sc) or intraperitoneal (ip) injections of the antigen and an adjuvant. It may be
30   useful to conjugate the antigen or a fragment containing the target amino acid sequence to
a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet
hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a
bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester
(conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues),
35   glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N \doteq C \doteq NR$, where R and R^1 are different
alkyl groups.

The route and schedule f the host animal r cultured antibody-pr ducing cells
therefrom are g nerally in keeping with established and c nventional t chniqu s for antibody

stimulation and production. While mice are frequently empl yed as the test model, it is
contemplated that any mammalian subject including human subjects or antibody-producing
cells obtained therefrom can be manipulated according to the processes of this invention to
serve as the basis for production of mammalian, including human, hybrid cell lines.

5   Animals are typically immunized against the immunogenic conjugates or derivatives by
combining 1 mg or 1 μg of conjugate (for rabbits or mice, respectively) with 3 volumes of
Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One
month later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in
Freund's complete adjuvant (or other suitable adjuvant) by subcutaneous injection at multiple

10   sites. 7 to 14 days later animals are bled and the serum is assayed for antigen titer. Animals
are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of
the same antigen, but conjugated to a different protein and/or through a different cross-linking
agent. Conjugates also can be made in recombinant cell culture as protein fusions. Also,
aggregating agents such as alum are used to enhance the immune response.

15   After immunization, monoclonal antibodies are prepared by recovering immune lymphoid
cells—typically spleen cells or lymphocytes from lymph node tissue—from immunized animals
and immortalizing the cells in conventional fashion, e.g. by fusion with myeloma cells or by
Epstein-Barr (EB)-virus transformation and screening for clones expressing the desired
antibody. The hybridoma technique described originally by Kohler and Milstein, *Eur. J.*

20   *Immunol.* 6:511 (1976) has been widely applied to produce hybrid cell lines that secrete high
levels of monoclonal antibodies against many specific antigens.

          It is possible to fuse cells of one species with another. However, it is preferable that
the source of the immunized antibody producing cells and the myeloma be from the same
species.

25   The hybrid cell lines can be maintained in culture *in vitro* in cell culture media. The cell
lines of this invention can be selected and/or maintained in a composition comprising the
continuous cell line in hypoxanthine-aminopterin thymidine (HAT) medium. In fact, once the
hybridoma cell line is established, it can be maintained on a variety of nutritionally adequate
media. Moreover, the hybrid cell lines can be stored and preserved in any number of

30   conventional ways, including freezing and storage under liquid nitrogen. Frozen cell lines can
be revived and cultured indefinitely with resumed synthesis and secretion of monoclonal
antibody. The secreted antibody is recovered from tissue culture supernatant by conventional
methods such as precipitation, ion exchange chromatography, affinity chromatography, or the
like. The antibodies described herein are also recovered from hybridoma cell cultures by

35   conventional methods for purification of IgG or IgM as the case may be that heretofore have
been used to purify these immunoglobulins from pooled plasma, e.g. ethanol or polyethylene
glycol precipitation procedur s. The purified antibodies are sterile filt red, and ptionally are

conjugated t a detectable marker such as an enzyme or spin label for use in diagnostic assays of the antigen in test samples.

While routinely rodent monoclonal antibodies are used as the source of the import antibody, the invention is not limited to any species. Additionally, techniques developed for

5   the production of chimeric antibodies (Morrison *et al.*, *Proc. Natl. Acad. Sci.*, 81:6851 (1984); Neuberger *et al.*, *Nature* 312:604 (1984); Takeda *et al.*, *Nature* 314:452 (1985)) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity (such as ability to activate human complement and mediate ADCC) can be used; such antibodies are within the

10   scope of this invention.

Techniques for creating recombinant DNA versions of the antigen-binding regions of antibody molecules (known as Fab fragments) which bypass the generation of monoclonal antibodies are encompassed within the practice of this invention. One extracts antibody-specific messenger RNA molecules from immune system cells taken from an immunized

15   animal, transcribes these into complementary DNA (cDNA), and clones the cDNA into a bacterial expressions system. One example of such a technique suitable for the practice of this invention was developed by researchers at Scripps/Stratagene, and incorporates a proprietary bacteriophage lambda vector system which contains a leader sequence that causes the expressed Fab protein to migrate to the periplasmic space (between the bacterial cell

20   membrane and the cell wall) or to be secreted. One can rapidly generate and screen great numbers of functional FAb fragments for those which bind the antigen. Such FAb fragments with specificity for the antigen are specifically encompassed within the term "antibody" as it is defined, discussed, and claimed herein.

25·-   Amino Acid Sequence Variants

Amino acid sequence variants of the antibodies and polypeptides of this invention (referred to in herein as the target polypeptide) are prepared by introducing appropriate nucleotide changes into the DNA encoding the target polypeptide, or by *in vitro* synthesis of the desired target polypeptide. Such variants include, for example, humanized variants of

30   non-human antibodies, as well as deletions from, or insertions or substitutions of, residues within particular amino acid sequences. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the target polypeptide, such as changing the number or position of

35   glycosylation sites, altering any membrane anchoring characteristics, and/or altering the intra-cellular location of the target polypeptide by inserting, deleting, or otherwise affecting any leader s quence of the native target polypeptide.

In designing amin acid sequence variants f target polypeptides, the location of the mutati n site and the nature f the mutation will depend on the target polypeptide characteristic(s) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more

5   radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting residues of the same or a different class adjacent to the located site, or combinations of options 1-3. In certain embodiments, these choices are guided by the methods for creating humanized sequences set forth above.

A useful method for identification of certain residues or regions of the target

10   polypeptide that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (Science, 244: 1081-1085 [1989]). Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the

15   surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to optimize the performance of a mutation at a given site, ala

20   scanning or random mutagenesis may be conducted at the target codon or region and the expressed target polypeptide variants are screened for the optimal combination of desired activity.

There are two principal variables in the construction of amino acid sequence variants: the location of the mutation site and the nature of the mutation. In general, the location and

25   nature of the mutation chosen will depend upon the target polypeptide characteristic to be modified.

Amino acid sequence deletions of antibodies are generally not preferred, as maintaining the generally configuration of an antibody is believed to be necessary for its activity. Any deletions will be selected so as to preserve the structure of the target antibody.

30   Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e., insertions within the target polypeptide sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5, most preferably 1 to 3. Examples of terminal insertions

35   include the target polypeptide with an N-terminal methionyl residue, an artifact of the direct expression of target polypeptide in bacterial recombinant cell culture, and fusion of a heterologous N-terminal signal sequence to the N-terminus of the target polypeptide molecule to facilitate the secr tion f the mature target polypeptide fr m recombinant host cells. Such

signal sequences gen rally will be obtained from, and thus hom logous to, the intended host cell species. Suitable sequences include STII or lpp for *E. coli*, alpha fact r for yeast, and viral signals such as herpes gD for mammalian cells.

Other insertional variants of the target polypeptide include the fusion to the N- or C-
5   terminus of the target polypeptide of immunogenic polypeptides, e.g., bacterial polypeptides such as beta-lactamase or an enzyme encoded by the *E. coli trp* locus, or yeast protein, and C-terminal fusions with proteins having a long half-life such as immunoglobulin constant regions (or other immunoglobulin regions), albumin, or ferritin, as described in WO 89/02922 published 6 April 1989.

10   Another group of variants are amino acid substitution variants. These variants have at least one amino acid residue in the target polypeptide molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as the active site(s) of the target polypeptide, and sites where the amino acids found in the target polypeptide from various species are substantially different
15   in terms of side-chain bulk, charge, and/or hydrophobicity. Other sites for substitution are described infra, considering the effect of the substitution of the antigen binding, affinity and other characteristics of a particular target antibody.

Other sites of interest are those in which particular residues of the target polypeptides obtained from various species are identical. These positions may be important for the
20   biological activity of the target polypeptide. These sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a relatively conservative manner. If such substitutions result in a change in biological activity, then other changes are introduced and the products screened until the desired effect is obtained.

Substantial modifications in function or immunological identity of the target polypeptide
25·   are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side chain properties:

30   (1) hydrophobic: norleucine, met, ala, val, leu, ile;
(2) neutral hydrophilic: cys, ser, thr;
(3) acidic: asp, glu;
(4) basic: asn, gln, his, lys, arg;
(5) residues that influence chain orientation: gly, pro; and
35   (6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another. Such substituted residues may be introduced into regions of the target

-29-

p lypeptide that are homologous with other antib dies of the same class or subclass, or, more preferably, int the n n-hom logous regi ns of the m lecule.

Any cysteine residues not involved in maintaining the proper conformation of target polypeptide also may be substituted, generally with serine, to improve the oxidative stability
5   of the molecule and prevent aberrant crosslinking.

DNA encoding amino acid sequence variants of the target polypeptide is prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR
10   mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the target polypeptide. A particularly preferred method of gene conversion mutagenesis is described below in Example 1. These techniques may utilized target polypeptide nucleic acid (DNA or RNA), or nucleic acid complementary to the target polypeptide nucleic acid.

Oligonucleotide-mediated mutagenesis is a preferred method for preparing substitution,
15   deletion, and insertion variants of target polypeptide DNA. This technique is well known in the art as described by Adelman et al., DNA, 2: 183 (1983). Briefly, the target polypeptide DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of the target polypeptide. After
20   hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the target polypeptide DNA.

Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the
25   template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as that described by Crea et al. (Proc. Natl. Acad. Sci. USA, 75: 5765 [1978]).

Single-stranded DNA template may also be generated by denaturing double-stranded
30   plasmid (or other) DNA using standard techniques.

For alteration of the native DNA sequence (to generate amino acid sequence variants, for example), the oligonucleotide is hybridized to the single-stranded template under suitable hybridization conditions. A DNA polymerizing enzyme, usually the Klenow fragment of DNA polymerase I, is then added to synthesize the complementary strand of the template using the
35   oligonucleotide as a primer for synthesis. A heteroduplex molecule is thus formed such that one strand of DNA encodes the mutated form of the target polypeptide, and the other strand (the original template) encodes the native, unaltered sequence of the target polypeptide. This heteroduplex molecule is then transf rmed int a suitable h st cell, usually a prokary te such

as *E. c li* JM101. After the cells are grown, they are plated onto agarose plates and screened using the olig nucle tide primer radi labeled with 32-ph sphat t identify the bacterial colonies that contain the mutated DNA. The mutated region is then removed and placed in an appropriate vector for protein production, generally an expression vector of the type
5   typically employed for transformation of an appropriate host.

The method described immediately above may be modified such that a homoduplex molecule is created wherein both strands of the plasmid contain the mutation(s). The modifications are as follows: The single-stranded oligonucleotide is annealed to the single-stranded template as described above. A mixture of three deoxyribonucleotides,
10 deoxyriboadenosine (dATP), deoxyriboguanosine (dGTP), and deoxyribothymidine (dTTP), is combined with a modified thio-deoxyribocytosine called dCTP-(aS) (which can be obtained from Amersham Corporation). This mixture is added to the template-oligonucleotide complex. Upon addition of DNA polymerase to this mixture, a strand of DNA identical to the template except for the mutated bases is generated. In addition, this new strand of DNA will contain
15 dCTP-(aS) instead of dCTP, which serves to protect it from restriction endonuclease digestion.

After the template strand of the double-stranded heteroduplex is nicked with an appropriate restriction enzyme, the template strand can be digested with ExoIII nuclease or another appropriate nuclease past the region that contains the site(s) to be mutagenized. The reaction is then stopped to leave a molecule that is only partially single-stranded. A complete
20 double-stranded DNA homoduplex is then formed using DNA polymerase in the presence of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase. This homoduplex molecule can then be transformed into a suitable host cell such as *E. coli* JM101, as described above.

DNA encoding target polypeptide variants with more than one amino acid to be substituted may be generated in one of several ways. If the amino acids are located close
25 together in the polypeptide chain, they may be mutated simultaneously using one oligonucleotide that codes for all of the desired amino acid substitutions. If, however, the amino acids are located some distance from each other (separated by more than about ten amino acids), it is more difficult to generate a single oligonucleotide that encodes all of the desired changes. Instead, one of two alternative methods may be employed.
30   In the first method, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid substitutions.

The alternative method involves two or more rounds of mutagenesis to produce the
35 desired mutant. The first round is as described for the single mutants: wild-type DNA is used for the template, an oligonucleotide encoding the first desired amino acid substitution(s) is annealed t this template, and the heteroduplex DNA m l cul is then generated. The second round of mutagen sis utilizes the mutated DNA produced in the first round of mutagenesis as

the template.   Thus, this template already contains   ne or more mutations.   The oligonucle tide nc ding the additional desir d amino acid substitution(s) is then annealed to this template, and the resulting strand of DNA now encodes mutations from both the first and second rounds of mutagenesis. This resultant DNA can be used as a template in a third round

5   of mutagenesis, and so on.

PCR mutagenesis is also suitable for making amino acid variants of target polypeptide. While the following discussion refers to DNA, it is understood that the technique also finds application with RNA. The PCR technique generally refers to the following procedure (see Erlich, *supra*, the chapter by R. Higuchi, p. 61-70): When small amounts of template DNA are

10   used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template. For introduction of a mutation into a plasmid DNA, one of the primers is designed to overlap the position of the mutation and to contain the mutation;

15   the sequence of the other primer must be identical to a stretch of sequence of the opposite strand of the plasmid, but this sequence can be located anywhere along the plasmid DNA. It is preferred, however, that the sequence of the second primer is located within 200 nucleotides from that of the first, such that in the end the entire amplified region of DNA bounded by the primers can be easily sequenced. PCR amplification using a primer pair like

20   the one just described results in a population of DNA fragments that differ at the position of the mutation specified by the primer, and possibly at other positions, as template copying is somewhat error-prone.

If the ratio of template to product material is extremely low, the vast majority of product DNA fragments incorporate the desired mutation(s). This product material is used to

25.   replace the corresponding region in the plasmid that served as PCR template using standard DNA technology. Mutations at separate positions can be introduced simultaneously by either using a mutant second primer, or performing a second PCR with different mutant primers and ligating the two resulting PCR fragments simultaneously to the vector fragment in a three (or more)-part ligation.

30   '   In a specific example of PCR mutagenesis, template plasmid DNA (1 μg) is linearized by digestion with a restriction endonuclease that has a unique recognition site in the plasmid DNA outside of the region to be amplified. Of this material, 100 ng is added to a PCR mixture containing PCR buffer, which contains the four deoxynucleotide tri-phosphates and is included in the GeneAmp® kits (obtained from Perkin-Elmer Cetus, Norwalk, CT and Emeryville, CA),

35   and 25 pmole of each oligonucleotide primer, to a final volume of 50 μl. The reaction mixture is overlayed with 35 μl mineral oil. The reaction is denatured for 5 minutes at 100°C, placed briefly n ice, and then 1 μl *Thermus aquaticus (Taq)* DNA polym rase (5 units/μl, purchased from Perkin-Elmer Cetus, Norwalk, CT and Emeryville, CA) is added bel w the mineral oil

layer. The reaction mixture is then ins rted int a DNA Thermal Cycler (purchased from
Perkin-Elmer Cetus) programmed as follows: 2 min. at 55°C, then 30 sec. at 72°C, then 19
cycles of the following: 30 sec. at 94°C, 30 sec. at 55°C, and 30 sec. at 72°C.

At the end of the program, the reaction vial is removed from the thermal cycler and the
5   aqueous phase transferred to a new vial, extracted with phenol/chloroform (50:50:vol), and
ethanol precipitated, and the DNA is recovered by standard procedures. This material is
subsequently subjected to the appropriate treatments for insertion into a vector.

Another method for preparing variants, cassette mutagenesis, is based on the technique
described by Wells et al. (Gene, 34: 315 [1985]). The starting material is the plasmid (or
10   other vector) comprising the target polypeptide DNA to be mutated. The codon(s) in the
target polypeptide DNA to be mutated are identified. There must be a unique restriction
endonuclease site on each side of the identified mutation site(s). If no such restriction sites
exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis
method to introduce them at appropriate locations in the target polypeptide DNA. After the
15   restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to
linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between
the restriction sites but containing the desired mutation(s) is synthesized using standard
procedures. The two strands are synthesized separately and then hybridized together using
standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This
20   cassette is designed to have 3' and 5' ends that are compatible with the ends of the linearized
plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the
mutated target polypeptide DNA sequence.

Insertion of DNA into a Cloning Vehicle

25·   The cDNA or genomic DNA encoding the target polypeptide is inserted into a replicable
vector for further cloning (amplification of the DNA) or for expression. Many vectors are
available, and selection of the appropriate vector will depend on 1) whether it is to be used
for DNA amplification or for DNA expression, 2) the size of the DNA to be inserted into the
vector, and 3) the host cell to be transformed with the vector. Each vector contains various
30   components depending on its function (amplification of DNA or expression of DNA) and the
host cell for which it is compatible. The vector components generally include, but are not
limited to, one or more of the following: a signal sequence, an origin of replication, one or
more marker genes, an enhancer element, a promoter, and a transcription termination
sequence.

35 .   (a) Signal Sequence Component

In general, the signal sequence may be a component of the vector, or it may be a part
of the target polypeptide DNA that is inserted into the vector.

The target polypeptides of this invention may be xpressed not only directly, but also as a fusi n with a heterol gous p lypeptide, preferably a signal sequence or other polyp ptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the

5   .target polypeptide DNA that is inserted into the vector. Included within the scope of this invention are target polypeptides with any native signal sequence deleted and replaced with a heterologous signal sequence. The heterologous signal sequence selected should be one that is recognized and processed (i.e. cleaved by a signal peptidase) by the host cell.   For prokaryotic host cells that do not recognize and process the native target polypeptide signal

10   sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native target polypeptide signal sequence may be substituted by the yeast invertase, alpha factor, or acid phosphatase leaders.   In mammalian cell expression the native signal sequence is satisfactory, although other

15   mammalian signal sequences may be suitable.

(b) Origin of Replication Component

       Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this

20   sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV

25   or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

       Most expression vectors are "shuttle" vectors, i.e. they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For

30   example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or . mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

       DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA

35   sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of the target polypeptide DNA. How ver, the recovery of genomic DNA encoding

the target polyp ptide is m re complex than that f an x genously replicated vector because restricti n enzyme digestion is required to excise the target polypeptide DNA.

(c) Selection Gene Component

5   Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g.

10   ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g. the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein

15   conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern *et al.*, J. Molec. Appl. Genet., 1: 327 [1982]), mycophenolic acid (Mulligan *et al.*, Science, 209: 1422 [1980]) or hygromycin (Sugden *et al.*, Mol. Cell. Biol., 5: 410-413 [1985]). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin

20   (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the target polypeptide nucleic acid, such as dihydrofolate reductase (DHFR) or thymidine kinase. The mammalian cell transformants are placed under selection pressure which only the transformants are uniquely

25   adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes the target polypeptide. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in

30   tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of the target polypeptide are synthesized from the amplified DNA.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed

35   is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77: 4216 [1980]. The transformed cells are then exposed to increased levels f methotrexate. This leads to the synthesis of multiple pies of the DHFR gene, and, concomitantly, multiple opi s of other

-35-

DNA c mprising th xpression vect rs, such as the DNA encoding the target polypeptid .
This amplification t chnique can be used with any oth rwise suitable host, .g., ATCC No.
CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant
DHFR gene that is highly resistant to Mtx is employed (EP 117,060). Alternatively, host cells
5   (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed
with DNA sequences encoding the target polypeptide, wild-type DHFR protein, and another
selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by
cell growth in medium containing a selection agent for the selectable marker such as an
aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Pat. No.
10   4,965,199.

A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid
YRp7 (Stinchcomb *et al.*, Nature, 282: 39 [1979]; Kingsman *et al.*, Gene, 7: 141 [1979]; or
Tschemper *et al.*, Gene, 10: 157 [1980]). The *trp*1 gene provides a selection marker for a
mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076
15   or PEP4-1 (Jones, Genetics, 85: 12 [1977]). The presence of the *trp*1 lesion in the yeast host
cell genome then provides an effective environment for detecting transformation by growth
in the absence of tryptophan. Similarly, *Leu*2-deficient yeast strains (ATCC 20,622 or
38,626) are complemented by known plasmids bearing the *Leu*2 gene.

20   (d) Promoter Component
Expression and cloning vectors usually contain a promoter that is recognized by the
host organism and is operably linked to the target polypeptide nucleic acid. Promoters are
untranslated sequences located upstream (5') to the start codon of a structural gene (generally
within about 100 to 1000 bp) that control the transcription and translation of a particular
25   nucleic acid sequence, such as that encoding the target polypeptide, to which they are
operably linked. Such promoters typically fall into two classes, inducible and constitutive.
Inducible promoters are promoters that initiate increased levels of transcription from DNA
under their control in response to some change in culture conditions, e.g. the presence or
absence of a nutrient or a change in temperature. At this time a large number of promoters
30   recognized by a variety of potential host cells are well known. These promoters are operably
linked to DNA encoding the target polypeptide by removing the promoter from the source DNA
by restriction enzyme digestion and inserting the isolated promoter sequence into the vector.
Both the native target polypeptide promoter sequence and many heterologous promoters may
be used to direct amplification and/or expression of the target polypeptide DNA. However,
35   heterologous promoters are preferred, as they generally permit greater transcription and higher
yields of expressed target polypeptide as compared to the native target polypeptide promoter.
Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose
prom ter systems (Chang *et al.*, Nature, 275: 615 [1978]; and Goedd l *et al.*, Nature, 281:

544 [1979]), alkaline ph sphatase, a trypt phan (trp) prom ter system (Goeddel, Nuclei Acids Res., 8: 4057 [1980] and EP 36,776) and hybrid promoters such as the tac promoter (deBoer et al., Proc. Natl. Acad. Sci. USA, 80: 21-25 [1983]). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby
5   enabling a skilled worker operably to ligate them to DNA encoding the target polypeptide (Siebenlist et al., Cell, 20: 269 [1980]) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also generally will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the target polypeptide.

Suitable promoting sequences for use with yeast hosts include the promoters for 3-
10   phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem., 255: 2073 [1980]) or other glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg., 7: 149 [1968]; and Holland, Biochemistry, 17: 4900 [1978]), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate
15   isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and
20   enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman et al., EP 73,657A. Yeast enhancers also are advantageously used with yeast promoters.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where
25. transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into mammalian expression vectors.
30   Target polypeptide transcription from vectors in mammalian host cells is controlled by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g. the actin promoter or
35   an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the target polypeptide sequence, provided such promoters are compatible with the host cell systems.

The early and late prom ters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replicati n. Fiers et al., Nature, 273:113 (1978); Mulligan and Berg, Science, 209: 1422-1427 (1980); Pavlakis et al., Proc. Natl. Acad. Sci. USA, 78: 7398-7402 (1981). The immediate early promoter of the human

5   cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Greenaway et al., Gene, 18: 355-360 (1982). A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. 4,419,446. A modification of this system is described in U.S. 4,601,978. See also Gray et al., Nature, 295: 503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; , Reyes et al., Nature, 297:

10   598-601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus, Canaani and Berg, Proc. Natl. Acad. Sci. USA, 79: 5166-5170 (1982) on expression of the human interferon β1 gene in cultured mouse and rabbit cells, and Gorman et al., Proc. Natl. Acad. Sci. USA, 79: 6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo

15   fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

(e) Enhancer Element Component

20   Transcription of DNA encoding the target polypeptide of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10-300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent having been found 5' (Laimins et al., Proc. Natl. Acad. Sci. USA, 78: 993 [1981]) and 3' (Lusky et

25· al., Mol. Cell Bio., 3: 1108 [1983]) to the transcription unit, within an intron (Banerji et al., Cell, 33: 729 [1983]) as well as within the coding sequence itself (Osborne et al., Mol. Cell Bio., 4: 1293 [1984]). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, σ-fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side

30   of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature, 297: 17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the target polypeptide DNA, but is preferably located at a site 5' from the promoter.

35

(f) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, r nucleated cells from ther multicellular organisms) will also contain sequences

-38-

necessary f r the terminati n f transcription and for stabilizing the mRNA. Such sequences are comm nly availabl from the 5' and, occasionally 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the target
5   polypeptide. The 3' untranslated regions also include transcription termination sites.

        Construction of suitable vectors containing one or more of the above listed components the desired coding and control sequences employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

10   For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform E. coli K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing et al., Nucleic Acids Res., 9: 309 (1981) or by the method of
15   Maxam et al., Methods in Enzymology, 65: 499 (1980).

        Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding the target polypeptide. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression
20   vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for
25   purposes of identifying analogs and variants of the target polypeptide that have target polypeptide-like activity.

        Other methods, vectors, and host cells suitable for adaptation to the synthesis of the target polypeptide in recombinant vertebrate cell culture are described in Gething et al., Nature, 293: 620-625 [1981]; Mantei et al., Nature, 281: 40-46 [1979]; Levinson et al.; EP
30   117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of the target polypeptide is pRK5 (EP pub. no. 307,247) or pSVI6B.

Selection and Transformation of Host Cells

        Suitable host cells for cloning or expressing the vectors herein are the prokaryote,
35   yeast, or higher eukaryote cells described above. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, for example, E. coli, Bacilli such as B. subtilis, Pseudomonas species such as P. aeruginosa, Salmonella typhimurium, or Serratia marcescans. One preferred E. coli cloning h st is E. coli 294 (ATCC 31,446), although other

strains such as *E. coli* B, *E. coli* χ1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325)
are suitable. These examples are illustrative rather than limiting. Preferably the host cell
should secrete minimal amounts of proteolytic enzymes. Alternatively, *in vitro* methods of
cloning, e.g. PCR or other nucleic acid polymerase reactions, are suitable.

5   In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are
suitable hosts for target polypeptide-encoding vectors. *Saccharomyces cerevisiae,* or common
baker's yeast, is the most commonly used among lower eukaryotic host microorganisms.
However, a number of other genera, species, and strains are commonly available and useful
herein, such as *Schizosaccharomyces pombe* [Beach and Nurse, Nature, 290: 140 (1981); EP
10   139,383 published May 2, 1985], *Kluyveromyces* hosts (U.S. 4,943,529) such as, e.g., *K.
lactis* [Louvencourt *et al.,* J. Bacteriol., 737 (1983)], *K. fragilis, K. bulgaricus, K.
thermotolerans,* and *K. marxianus, yarrowia* [EP 402,226], *Pichia pastoris* [EP 183,070;
Sreekrishna *et al.,* J. Basic Microbiol., 28: 265-278 (1988)], *Candida, Trichoderma reesia* [EP
244,234], *Neurospora crassa* [Case *et al.,* Proc. Natl. Acad. Sci. USA, 76: 5259-5263
15   (1979)], and filamentous fungi such as, e.g, *Neurospora, Penicillium, Tolypocladium* [WO
91/00357 published 10 January 1991], and *Aspergillus* hosts such as *A. nidulans* [Ballance
et al., Biochem. Biophys. Res. Commun., 112: 284-289 (1983); Tilburn *et al.,* Gene, 26: 205-
221 (1983); Yelton *et al.,* Proc. Natl. Acad. Sci. USA, 81: 1470-1474 (1984)] and *A. niger*
[Kelly and Hynes, EMBO J., 4: 475-479 (1985)].

20   Suitable host cells for the expression of glycosylated target polypeptide are derived
from multicellular organisms. Such host cells are capable of complex processing and
glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether
from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect
cells. Numerous baculoviral strains and variants and corresponding permissive insect host
25   cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes
albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* host cells have
been identified. See, e.g., Luckow *et al.,* Bio/Technology, 6: 47-55 (1988); Miller *et al.,* In
Genetic Engineering, Setlow, J.K. *et al.,* eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279;
and Maeda *et al.,* Nature, 315: 592-594 (1985). A variety of such viral strains are publicly
30   available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx
mori* NPV, and such viruses may be used as the virus herein according to the present
invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of
cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts.
Typically, plant cells are transfected by incubation with certain strains of the bacterium
35   *Agrobacterium tumefaciens,* which has been previously manipulated to contain the target
polypeptide DNA. During incubation of the plant cell culture with *A. tumefaciens,* the DNA
encoding target polypeptide is transferred to the plant cell host such that it is transfected, and
will, under appropriat c nditions, express the target polypeptide DNA. In addition, regulatory

and signal sequenc s compatible with plant cells are available, such as th nopaline synthase promoter and p lyad nylati n signal sequences. Depicker *et al.*, J. Mol. Appl. G n., 1: 561 (1982). In additi n, DNA segments is lated fr m the upstream regi n of the T-DNA *780* gene are capable of activating or increasing transcription levels of plant-expressible genes in
5   recombinant DNA-containing plant tissue. See EP 321,196 published 21 June 1989.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years [Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)]. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human
10   embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, J. Gen Virol., 36: 59 [1977]); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77: 4216 [1980]); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23: 243-251 [1980]); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587);
15   human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, Annals N.Y. Acad. Sci., 383: 44-68 [1982]); MRC 5 cells; FS4 cells; and a human hepatoma cell line (Hep G2). Preferred host cells are human
20   embryonic kidney 293 and Chinese hamster ovary cells.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

25   Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

30   Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook *et al.*, *supra*, is generally used for prokaryotes or other cells that contain substantial cell-wall
35   barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23: 315 (1983) and WO 89/05859 published 29 June 1989. For mammalian cells without such cell walls, the calcium phosphate precipitation method described in sections 16.30-16.37 of Sambr ok *et al*, *supra*, is preferred. General

aspects of mammalian cell h st system transformations have been described by Axel in U.S. 4,399,216 issu d 16 August 1983. Transformati ns into yeast are typically carried out according to the method of Van Solingen et al., J. Bact., 130: 946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76: 3829 (1979). However, other methods for introducing DNA

5   into cells such as by nuclear injection, electroporation, or protoplast fusion may also be used.

Culturing the Host Cells

Prokaryotic cells used to produce the target polypeptide of this invention are cultured in suitable media as described generally in Sambrook et al., supra.

10   The mammalian host cells used to produce the target polypeptide of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, Meth. Enz., 58: 44 (1979), Barnes

15   and Sato, Anal. Biochem., 102: 255 (1980), U.S. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Pat. Re. 30,985, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such

20   as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are

25   those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The host cells referred to in this disclosure encompass cells in in vitro culture as well as cells that are within a host animal.

It is further envisioned that the target polypeptides of this invention may be produced
30   by homologous recombination, or with recombinant production methods utilizing control elements introduced into cells already containing DNA encoding the target polypeptide currently in use in the field. For example, a powerful promoter/enhancer element, a suppressor, or an exogenous transcription modulatory element is inserted in the genome of the intended host cell in proximity and orientation sufficient to influence the transcription of

35   DNA encoding the desired target polypeptide. The control element does not encode the target polypeptide of this invention, but the DNA is present in the host cell genome. One next screens for cells making the target polypeptide of this invention, or increased or decreased levels of expressi n, as desired.

Detecting Gene Amplification/Expression

G ne amplificati n and/ r expressi n may be measured In a sample dir ctly, for example, by conventional Southern blotting, northern blotting to quantitate the transcription of mRNA (Thomas, Proc. Natl. Acad. Sci. USA, 77: 5201-5205 [1980]), dot blotting (DNA
5   analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such
10   as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, Including DNA duplexes; RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.
15   Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With Immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are
20   usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hsu *et al.*, Am. J. Clin. Path., 75: 734-738 (1980).

Antibodies useful for Immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared In any mammal. Conveniently, the
25   antibodies may be prepared against a native target polypeptide or against a synthetic peptide based on the DNA sequences provided herein as described further in Section 4 below.

Purification of The Target polypeptide

The target polypeptide preferably is recovered from the culture medium as a secreted
30   polypeptide, although it also may be recovered from host cell lysates when directly expressed without a secretory signal.

When the target polypeptide is expressed In a recombinant cell other than one of human origin, the target polypeptide is completely free of proteins or polypeptides of human origin. However, it is necessary to purify the target polypeptide from recombinant cell proteins or
35   polypeptides to obtain preparations that are substantially homogeneous as to the target polypeptide. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. The target polypeptide may then be purified from the s luble protein fraction and fr m the membrane

-43-

fraction of the culture lysate, depending on whether the target polypeptide is membrane bound. The foll wing pr cedures are exemplary of suitable purification pr cedures: fractionati n on immun affinity r ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation exchange resin such as DEAE;
5   chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

Target polypeptide variants in which residues have been deleted, inserted or substituted are recovered in the same fashion, taking account of any substantial changes in properties
10   occasioned by the variation. For example, preparation of a target polypeptide fusion with another protein or polypeptide, e.g. a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing antibody to the antigen (or containing antigen, where the target polypeptide is an antibody) can be used to adsorb the fusion. Immunoaffinity columns such as a rabbit polyclonal anti-target polypeptide column can be employed to absorb the
15   target polypeptide variant by binding it to at least one remaining immune epitope. A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native target polypeptide may require modification to account for
20   changes in the character of the target polypeptide or its variants upon expression in recombinant cell culture.

Covalent Modifications of Target Polypeptides

Covalent modifications of target polypeptides are included within the scope of this
25   Invention. One type of covalent modification included within the scope of this invention is a target polypeptide fragment. Target polypeptide fragments having up to about 40 amino acid residues may be conveniently prepared by chemical synthesis, or by enzymatic or chemical cleavage of the full-length target polypeptide or variant target polypeptide. Other types of covalent modifications of the target polypeptide or fragments thereof are introduced into the
30   molecule by reacting specific amino acid residues of the target polypeptide or fragments thereof with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues.

Cysteinyl residues most commonly are reacted with σ-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or
35   carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, σ-bromo-β-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-

chloromercuribenzoate, 2-chl r m rcuri-4-nitr phenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay, the chloramine T method described above being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides ($R'-N=C=N-R'$), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking target polypeptide to a water-insoluble support matrix or surface for use in the method for purifying anti-target polypeptide antibodies, and vice versa. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alt rnatively, reactive water-insoluble matrices such as cyanogen bromide-

activated carb hydrates and the reactive substrates described in U.S. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. Alternatively, these residues are deamidated

5   under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecular

10   Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 [1983]), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.   Another   type   of covalent modification of the target polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. By altering is meant deleting one or more carbohydrate moieties found in the native target polypeptide, and/or

15   adding one or more glycosylation sites that are not present in the native target polypeptide.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tri-peptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the

20   carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tri-peptide sequences in a polypeptide creates a potential glycosylation site.   O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose, to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

25   Addition of glycosylation sites to the target polypeptide is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tri-peptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native target polypeptide sequence (for O-linked glycosylation sites).   For ease, the target

30   polypeptide amino acid sequence is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the target polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids.   The DNA mutation(s) may be made using methods described above under the heading of "Amino Acid Sequence Variants of Target Polypeptide".

35   Another means of increasing the number of carbohydrate moieties on the target polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. These procedures are advantageous in that they do not require production f the polypeptide in a h st cell that has glycosylation capabilities for N- and O- link d glycosylati n. Depending on

the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl gr ups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) ar matic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods

5   are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston (CRC Crit. Rev. Biochem., pp. 259-306 [1981]).

Removal of carbohydrate moieties present on the native target polypeptide may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This

10   treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin et al. (Arch. Biochem. Biophys., 259:52 [1987]) and by Edge et al. (Anal. Biochem., 118:131 [1981]). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-

15   glycosidases as described by Thotakura et al. (Meth. Enzymol., 138:350 [1987]).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin et al. (J. Biol. Chem., 257:3105 [1982]). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of the target polypeptide comprises linking the

20   target polypeptide to various nonproteinaceous polymers, e.g. polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the manner set forth in U.S. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

The target polypeptide also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example,

25   hydroxymethylcellulose or gelatin-microcapsules and poly-[methylmethacylate] microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th edition, Osol, A., Ed., (1980).

30   Target polypeptide preparations are also useful in generating antibodies, for screening for binding partners, as standards in assays for the target polypeptide (e.g. by labeling the target polypeptide for use as a standard in a radioimmunoassay, enzyme-linked immunoassay, or radioreceptor assay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with radioiodine, enzymes, fluorophores, spin labels, and the like.

35   Since it is often difficult to predict in advance the characteristics of a variant target polypeptide, it will be appreciated that some screening of the recovered variant will be needed to select the optimal variant. For example, a change in the immunological character of the target polypeptide molecule, such as affinity for a given antig n or antibody, is m asured by

-47-

a comp titive-typ immun assay. The variant is assay d f r changes in the suppression or enhan ment of its activity by c mparis n t the activity bserved for th target polypeptide in the same assay. Other potential modifications of pr tein or polypeptide properties such as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, stability in recombinant cell culture or in plasma, or the tendency to aggregate with carriers or into multimers are assayed by methods well known in the art.

Diagnostic and Related Uses of the Antibodies

The antibodies of this invention are useful in diagnostic assays for antigen expression in specific cells or tissues. The antibodies are detectably labeled and/or are immobilized on an insoluble matrix.

The antibodies of this invention find further use for the affinity purification of the antigen from recombinant cell culture or natural sources. Suitable diagnostic assays for the antigen and its antibodies depend on the particular antigen or antibody. Generally, such assays include competitive and sandwich assays, and steric inhibition assays. Competitive and sandwich methods employ a phase-separation step as an integral part of the method while steric inhibition assays are conducted in a single reaction mixture. Fundamentally, the same procedures are used for the assay of the antigen and for substances that bind the antigen, although certain methods will be favored depending upon the molecular weight of the substance being assayed. Therefore, the substance to be tested is referred to herein as an analyte, irrespective of its status otherwise as an antigen or antibody, and proteins that bind to the analyte are denominated binding partners, whether they be antibodies, cell surface receptors, or antigens.

Analytical methods for the antigen or its antibodies all use one or more of the following reagents: labeled analyte analogue, immobilized analyte analogue, labeled binding partner, immobilized binding partner and steric conjugates. The labeled reagents also are known as "tracers."

The label used (and this is also useful to label antigen nucleic acid for use as a probe) is any detectable functionality that does not interfere with the binding of analyte and its binding partner. Numerous labels are known for use in immunoassay, examples including moieties that may be detected directly, such as fluorochrome, chemiluminescent, and radioactive labels, as well as moieties, such as enzymes, that must be reacted or derivatized to be detected. Examples of such labels include the radioisotopes ^{32}P, ^{14}C, ^{125}I, ^{3}H, and ^{131}I, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luceriferases, e.g., firefly luciferase and bacterial luciferase (U.S. Pat. No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g., glucose xidase, galactose oxidase, and gluc se-6-phosphate dehydrogenas ,

heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide t   xidize a dye pr cursor such as HRP, lactoperoxidase, or microperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free radicals, and the like.

5   Conventional methods are available to bind these labels covalently to proteins or polypeptides. For instance, coupling agents such as dialdehydes, carbodiimides, dimaleimides, bis-imidates, bis-diazotized benzidine, and the like may be used to tag the antibodies with the above-described fluorescent, chemiluminescent, and enzyme labels. See, for example, U.S. Pat. Nos. 3,940,475 (fluorimetry) and 3,645,090 (enzymes); Hunter et al., Nature, 144: 945

10   (1962); David et al., Biochemistry, 13: 1014-1021 (1974); Pain et al., J. Immunol. Methods, 40: 219-230 (1981); and Nygren, J. Histochem. and Cytochem., 30: 407-412 (1982). Preferred labels herein are enzymes such as horseradish peroxidase and alkaline phosphatase. The conjugation of such label, including the enzymes, to the antibody is a standard manipulative procedure for one of ordinary skill in immunoassay techniques. See, for

15   example, O'Sullivan et al., "Methods for the Preparation of Enzyme-antibody Conjugates for Use in Enzyme Immunoassay," in Methods in Enzymology, ed. J.J. Langone and H. Van Vunakis, Vol. 73 (Academic Press, New York, New York, 1981), pp. 147-166. Such bonding methods are suitable for use with the antibodies and polypeptides of this invention.

          Immobilization of reagents is required for certain assay methods. Immobilization entails

20   separating the binding partner from any analyte that remains free in solution. This conventionally is accomplished by either insolubilizing the binding partner or analyte analogue before the assay procedure, as by adsorption to a water-insoluble matrix or surface (Bennich et al., U.S. 3,720,760), by covalent coupling (for example, using glutaraldehyde cross-linking), or by insolubilizing the partner or analogue afterward, e.g., by immunoprecipitation.

25   Other assay methods, known as competitive or sandwich assays, are well established and widely used in the commercial diagnostics industry.

          Competitive assays rely on the ability of a tracer analogue to compete with the test sample analyte for a limited number of binding sites on a common binding partner. The binding partner generally is insolubilized before or after the competition and then the tracer

30   and analyte bound to the binding partner are separated from the unbound tracer and analyte. This separation is accomplished by decanting (where the binding partner was preinsolubilized) or by centrifuging (where the binding partner was precipitated after the competitive reaction). The amount of test sample analyte is inversely proportional to the amount of bound tracer as measured by the amount of marker substance. Dose-response curves with known amounts

35   of analyte are prepared and compared with the test results to quantitatively determine the amount of analyte present in the test sample. These assays are called ELISA systems when enzymes are used as the detectable markers.

Another speci s of competitiv assay, called a "h mogeneous" assay, d es not require a phase separati n. Here, a c njugate of an enzyme with the analyt is prepared and used such that when anti-analyte binds to the analyte the presence of the anti-analyte modifies the enzyme activity. In this case, the antigen or its immunologically active fragments are

5   conjugated with a bifunctional organic bridge to an enzyme such as peroxidase. Conjugates are selected for use with antibody so that binding of the antibody inhibits or potentiates the enzyme activity of the label. This method *per se* is widely practiced under the name of EMIT.

Steric conjugates are used in steric hindrance methods for homogeneous assay. These conjugates are synthesized by covalently linking a low-molecular-weight hapten to a small

10   analyte so that antibody to hapten substantially is unable to bind the conjugate at the same time as anti-analyte. Under this assay procedure the analyte present in the test sample will bind anti-analyte, thereby allowing anti-hapten to bind the conjugate, resulting in a change in the character of the conjugate hapten, e.g., a change in fluorescence when the hapten is a fluorophore.

15   Sandwich assays particularly are useful for the determination of antigen or antibodies. In sequential sandwich assays an immobilized binding partner is used to adsorb test sample analyte, the test sample is removed as by washing, the bound analyte is used to adsorb labeled binding partner, and bound material is then separated from residual tracer. The amount of bound tracer is directly proportional to test sample analyte. In "simultaneous"

20   sandwich assays the test sample is not separated before adding the labeled binding partner. A sequential sandwich assay using an anti-antigen monoclonal antibody as one antibody and a polyclonal anti-antigen antibody as the other is useful in testing samples for particular antigen activity.

The foregoing are merely exemplary diagnostic assays for the import and humanized

25   antibodies of this invention. Other methods now or hereafter developed for the determination of these analytes are included within the scope hereof, including the bioassays described above.

Immunotoxins

30   This invention is also directed to immunochemical derivatives of the antibodies of this invention such as immunotoxins (conjugates of the antibody and a cytotoxic moiety). Antibodies which carry the appropriate effector functions, such as with their constant domains, are also used to induce lysis through the natural complement process, and to interact with antibody dependent cytotoxic cells normally present.

35   For example, purified, sterile filtered antibodies are optionally conjugated to a cytotoxin such as ricin for use in AIDS therapy. European Publication No. 279688 illustrates methods f r making and using immunotoxins for the treatment of HIV infection. The methods of this

invention, for example, are suitable for obtaining humanized antibodies for use as immunotoxins for use in AIDS therapy.

The cytotoxic moiety of the immunotoxin may be a cytotoxic drug or an enzymatically active toxin of bacterial, fungal, plant or animal origin, or an enzymatically active fragment of

5   such a toxin. Enzymatically active toxins and fragments thereof used are diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin,

10   restrictocin, phenomycin, enomycin and the tricothecenes. In another embodiment, the antibodies are conjugated to small molecule anticancer drugs such as cis-platin or 5FU. Conjugates of the monoclonal antibody and such cytotoxic moieties are made using a variety of bifunctional protein coupling agents. Examples of such reagents are SPDP, IT , bifunctional derivatives of imidoesters such as dimethyl adipimidate HCl, active esters such as

15   disuccinimidyl suberate, aldehydes such as glutaraldehyde, bis-azido compounds such as bis (p-azidobenzoyl) hexanediamine, bis-diazonium derivatives such as bis- (p-diazoniumbenzoyl)-ethylenediamine, diisocyanates such as tolylene 2,6-diisocyanate and bis-active fluorine compounds such as 1,5-difluoro- 2,4-dinitrobenzene. The lysing portion of a toxin may be joined to the Fab fragment of the antibodies.

20   Immunotoxins can be made in a variety of ways, as discussed herein. Commonly known crosslinking reagents can be used to yield stable conjugates.

Advantageously, monoclonal antibodies specifically binding the domain of the antigen which is exposed on the infected cell surface, are conjugated to ricin A chain. Most advantageously the ricin A chain is deglycosylated and produced through recombinant means.

25   An advantageous method of making the ricin immunotoxin is described in Vitetta *et al.*, *Science* 238:1098 (1987).

When used to kill infected human cells *in vitro* for diagnostic purposes, the conjugates will typically be added to the cell culture medium at a concentration of at least about 10 nM. The formulation and mode of administration for *in vitro* use are not critical. Aqueous

30   formulations that are compatible with the culture or perfusion medium will normally be used. Cytotoxicity may be read by conventional techniques.

Cytotoxic radiopharmaceuticals for treating infected cells may be made by conjugating radioactive isotopes (e.g. I, Y, Pr) to the antibodies. Advantageously alpha particle-emitting isotopes are used. The term 'cytotoxic moiety° as used herein is intended to include such

35   isotopes.

In a preferred embodiment, ricin A chain is deglycosylated or produced without oligosaccharides, to decrease its clearance by irrelevant clearance mechanisms (e.g., the liver).

In another embodiment, whole ricin (A hain plus B chain) is conjugated to antibody if the galact se binding property of B-chain can be blocked ("bl cked ricin").

In a further embodiment toxin-conjugates are made with Fab or F(ab')$_2$ fragm nts. Because of their relatively small size these fragments can better penetrate tissue to reach

5   infected cells.

In another embodiment, fusogenic liposomes are filled with a cytotoxic drug and the liposomes are coated with antibodies specifically binding the particular antigen.

Antibody Dependent Cellular Cytotoxicity

Certain aspects of this invention involve antibodies which are (a) directed against a

10   particular antigen and (b) belong to a subclass or isotype that is capable of mediating the lysis of cells to which the antibody molecule binds. More specifically, these antibodies should belong to a subclass or isotype that, upon complexing with cell surface proteins, activates serum complement and/or mediates antibody dependent cellular cytotoxicity (ADCC) by activating effector cells such as natural killer cells or macrophages.

15   Biological activity of antibodies is known to be determined, to a large extent, by the constant domains or Fc region of the antibody molecule (Uananue and Benacerraf, *Textbook of Immunology*, 2nd Edition, Williams & Wilkins, p. 218 (1984)). This includes their ability to activate complement and to mediate antibody-dependent cellular cytotoxicity (ADCC) as effected by leukocytes. Antibodies of different classes and subclasses differ in this respect,

20   as do antibodies from the same subclass but different species; according to the present invention, antibodies of those classes having the desired biological activity are prepared. Preparation of these antibodies involves the selection of antibody constant domains are their incorporation in the humanized antibody by known technique. For example, mouse immunoglobulins of the IgG3 and IgG2a class are capable of activating serum complement

25   upon binding to the target cells which express the cognate antigen, and therefore humanized antibodies which incorporate IgG3 and IgG2a effector functions are desirable for certain therapeutic applications.

In general, mouse antibodies of the IgG2a and IgG3 subclass and occasionally IgG1 can mediate ADCC, and antibodies of the IgG3, IgG2a, and IgM subclasses bind and activate

30   serum complement. Complement activation generally requires the binding of at least two IgG molecules in close proximity on the target cell. However, the binding of only one IgM molecule activates serum complement.

The ability of any particular antibody to mediate lysis of the target cell by complement activation and/or ADCC can be assayed. The cells of interest are grown and labeled *in vitro*;

35   the antibody is added to the cell culture in combination with either serum complement or immune cells which may be activated by the antigen antibody complexes. Cytolysis of the target cells is detected by the release of label from the lysed cells. In fact, antibodies can be screened using the patient's own serum as a source of complement and/or immune cells. The

antibody that is capable of activating complement or mediating ADCC in the *in vitro* test can th n b used therapeutically in that particular patient.

This invention specifically encompasses consensus Fc antibody d mains prepared and used according to the teachings of this invention.

5

Therapeutic and Other Uses of the Antibodies

When used *in vivo* for therapy, the antibodies of the subject invention are administered to the patient in therapeutically effective amounts (i.e. amounts that have desired therapeutic effect). They will normally be administered parenterally. The dose and dosage regimen will

10 depend upon the degree of the infection, the characteristics of the particular antibody or immunotoxin used, e.g., its therapeutic index, the patient, and the patient's history. Advantageously the antibody or immunotoxin is administered continuously over a period of 1-2 weeks, intravenously to treat cells in the vasculature and subcutaneously and intraperitoneally to treat regional lymph nodes. Optionally, the administration is made during

15 the course of adjunct therapy such as combined cycles of radiation, chemotherapeutic treatment, or administration of tumor necrosis factor, interferon or other cytoprotective or immunomodulatory agent.

For parenteral administration the antibodies will be formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically

20 acceptable parenteral vehicle. Such vehicles are inherently nontoxic, and non-therapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils and ethyl oleate can also be used. Liposomes may be used as carriers. The vehicle may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, e.g., buffers and

25 preservatives. The antibodies will typically be formulated in such vehicles at concentrations of about 1 mg/ml to 10 mg/ml.

Use of IgM antibodies may be preferred for certain applications, however IgG molecules by being smaller may be more able than IgM molecules to localize to certain types of infected cells.

30 There is evidence that complement activation *in vivo* leads to a variety of biological effects, including the induction of an inflammatory response and the activation of macrophages (Uananue and Benecerraf, *Textbook of Immunology*, 2nd Edition, Williams & Wilkins, p. 218 (1984)). The increased vasodilation accompanying inflammation may increase the ability of various agents to localize in infected cells. Therefore, antigen-antibody

35 combinations of the type specified by this invention can be used therapeutically in many ways. Additionally, purified antigens (Hakomori, *Ann. Rev. Immunol.* 2:103 (1984)) or anti-idiotypic antibodies (Nepom *et al.*, *Proc. Natl. Acad. Sci.* 81:2864 (1985); Koprowski *et al.*, *Proc. Natl. Acad. Sci.* 81:216 (1984)) relating to such antigens could be used to induce

-53-

an active immune resp nse in human patients. Such a response includes the f rmation of antib dies capable of activating human comploment and mediating ADCC and by such mechanisms cause infected cell destruction.

Optionally, the antibodies of this invention are useful in passively immunizing patients,
5   as exemplified by the administration of humanized anti-HIV antibodies.

The antibody compositions used in therapy are formulated and dosages established in a fashion consistent with good medical practice taking into account the disorder to be treated, the condition of the individual patient, the site of delivery of the composition, the method of administration and other factors known to practitioners. The antibody compositions are
10   prepared for administration according to the description of preparation of polypeptides for administration, *infra*.

Deposit of Materials

As described above, cultures of the muMAb4D5 have been deposited with the
15   American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC).

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of viable cultures for 30 years from the date of the deposit. The organisms will be made available by ATCC under the
20   terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the cultures to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be
25   entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.12 with particular reference to 886 OG 638).

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or
30   withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

The assignee of the present application has agreed that if the cultures on deposit should die or be lost or destroyed when cultivated under suitable conditions, they will be promptly replaced on notification with a viable specimen of the same culture. Availability of the
35   deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable ne skilled in the art t practice the inventi n. Th pr sent invention is n t t be limited in scope by th constructs deposited, since the deposit d embodiments are int nded to illustrate only certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that they represent. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

It is understood that the application of the teachings of the present invention to a specific problem or situation will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples of the products of the present invention and representative processes for their isolation, use, and manufacture appear below, but should not be construed to limit the invention.

EXAMPLES

EXAMPLE 1. HUMANIZATION OF muMAb4D5

Here we report the chimerization of muMAb4D5 (chMAb4D5) and the rapid and simultaneous humanization of heavy (V_H) and light (V_L) chain variable region genes using a novel "gene conversion mutagenesis" strategy. Eight humanized variants (huMAb4D5) were constructed to probe the importance of several FR residues identified by our molecular modeling or previously proposed to be critical to the conformation of particular CDRs (see Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901-917 (1987); Chothia, C. *et al., Nature* 342:877-883 (1989); Tramontano, A. *et al., J. Mol. Biol.* 215:175-182 (1990)). Efficient transient expression of humanized variants in non-myeloma cells allowed us to rapidly investigate the relationship between binding affinity for p185^{HER2} ECD and anti-proliferative activity against p185^{HER2} overexpressing carcinoma cells.

MATERIALS and METHODS

Cloning of Variable Region Genes. The muMAb4D5 V_H and V_L genes were isolated by polymerase chain reaction (PCR) amplification of mRNA from the corresponding hybridoma (Fendly, B. M. *et al., Cancer Res.* 50:1550-1558 (1990)) as described by Orlandi et al. (Orlandi, R. *et al., Proc. Natl. Acad. Sci. USA* 86:3833-3837 (1989)). Amino terminal sequencing of muMAb4D5 V_L and V_H was used to design the sense strand PCR primers, whereas the anti-sense PCR primers were based upon consensus sequences of murine framework residues (Orlandi, R. *et al., Proc. Natl. Acad. Sci. USA* 86:3833-3837 (1989); Kabat, E. A. *et al., Sequences of Proteins of Immunological Interest* (National Institutes of

-55-

Health, Bethesda, MD, 1987)) incorporating restriction sites f r directional cloning shown by underlining and listed after the sequen es: V_L sense, 5'-TCC<u>GATATC</u>CAGCTGACCCAGTCTCCA-3' (SEQ. ID NO. 7), EcoRV; V_L anti-sense, 5'-GTTTGATCTCCAGCTT<u>GGTACC</u>HSCDCCGAA-3' (SEQ. ID NO. 8), Asp718; V_H sense, 5'-

5   AGGTSMAR<u>CTGCAG</u>SAGTCWGG-3' (SEQ. ID NO. 9), Pstl and V_H anti-sense, 5'-TGAGGAGAC<u>GGTGACC</u>GTGGTCCCTTGGCCCCAG-3' (SEQ. ID NO. 10), BstEII; where H = A or C or T, S = C or G, D = A or G or T, M = A or C, R = A or G and W = A or T. The PCR products were cloned into pUC119 (Vieira, J. & Messing, J., Methods Enzymol. 153:3-11 (1987)) and five clones for each variable domain sequenced by the dideoxy method

10   (Sanger, F. et al., Proc. Natl. Acad. Sci. USA 74:5463-5467 (1977)).

Molecular Modelling. Models for muMAb4D5 V_H and V_L domains were constructed separately from consensus coordinates based upon seven Fab structures from the Brookhaven protein data bank (entries 1FB4, 2RHE, 2MCP, 3FAB, 1FBJ, 2HFL and 1REI). The Fab fragment KOL (Marquart, M. et al., J. Mol. Biol. 141:369-391 (1980)) was first chosen as a

15   template for V_L and V_H domains and additional structures were then superimposed upon this structure using their main chain atom coordinates (INSIGHT program, Biosym Technologies). The distance from the template $C\alpha$ to the analogous $C\alpha$ in each of the superimposed structures was calculated for each residue position. If all (or nearly all) $C\alpha$-$C\alpha$ distances for a given residue were ≤ 1Å, then that position was included in the consensus structure. In

20   most cases the β-sheet framework residues satisfied these criteria whereas the CDR loops did not. For each of these selected residues the average coordinates for individual N, $C\alpha$, C, O and $C\beta$ atoms were calculated and then corrected for resultant deviations from non-standard bond geometry by 50 cycles of energy minimization using the DISCOVER program (Biosym Technologies) with the AMBER forcefield (Weiner, S. J. et al., J. Amer. Chem. Soc.

25   106:765-784 (1984)) and $C\alpha$ coordinates fixed. The side chains of highly conserved residues, such as the disulfide-bridged cysteine residues, were then incorporated into the resultant consensus structure. Next the sequences of muMAb4D5 V_L and V_H were incorporated starting with the CDR residues and using the tabulations of CDR conformations from Chothia et al. (Chothia, C. et al., Nature 342:877-883 (1989)) as a guide. Side-chain conformations

30   were chosen on the basis of Fab crystal structures, rotamer libraries (Ponder, J. W. & Richards, F. M., J. Mol. Biol. 193:775-791 (1987)) and packing considerations. Since V_H-CDR3 could not be assigned a definite backbone conformation from these criteria, two models were created from a search of similar sized loops using the INSIGHT program. A third model was derived using packing and solvent exposure considerations. Each model was then

35   subjected to 5000 cycles of energy minimization.

In humanizing muMAb4D5, consensus human sequences were first derived from the most abundant subclasses in the sequence compilation of Kabat et al. (Kabat, E. A. et al., Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, MD,

-56-

1987)), nam ly V_L κ subgr up I and V_H group III, and a molecular model generated for these sequences using the methods described above. A structur for huMAb4D5 was created by transferring the CDRs from the muMAb4D5 m del into the c nsensus human structure. All huMAb4D5 variants contain human replacements of muMAb4D5 residues at three positions

5   within CDRs as defined by sequence variability (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987)) but not as defined by structural variability (Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901-917 (1987)): V_L-CDR1 K24R, V_L-CDR2 R54L and V_L-CDR2 T56S. Differences between muMAb4D5 and the human consensus framework residues (Fig. 1) were individually modeled to investigate

10   their possible influence on CDR conformation and/or binding to the p185^{HER2} ECD.

Construction of Chimeric Genes. Genes encoding chMAb4D5 light and heavy chains were separately assembled in previously described phagemid vectors containing the human cytomegalovirus enhancer and promoter, a 5' intron and SV40 polyadenylation signal (Gorman, C. M. et al., *DNA & Prot. Engin. Tech.* 2:3-10 (1990)). Briefly, gene segments

15   encoding muMAb4D5 V_L (Fig. 1A) and REI human κ_1 light chain C_L (Palm, W. & Hilschmann, N., *Z. Physiol. Chem.* 356:167-191 (1975)) were precisely joined as were genes for muMAb4D5 V_H (Fig. 1B) and human γ1 constant region (Capon, D. J. et al., *Nature* 337:525-531 (1989)) by simple subcloning (Boyle, A., in *Current Protocols in Molecular Biology*, Chapter 3 (F. A. Ausubel et al., eds., Greene Publishing & Wiley-Interscience, New

20   York, 1990)) and site-directed mutagenesis (Carter, P., in *Mutagenesis: A Practical Approach*, Chapter 1 (IRL Press, Oxford, UK 1991)). The γ1 isotype was chosen as it has been found to be the preferred human isotype for supporting ADCC and complement dependent cytotoxicity using matched sets of chimeric (Brüggemann, M. et al., *J. Exp. Med.* 166:1351-1361 (1987)) or humanized antibodies (Riechmann, L. et al., *Nature* 332:323-327

25   (1988)). The PCR-generated V_L and V_H fragments (Fig. 1) were subsequently mutagenized so that they faithfully represent the sequence of muMAb4D5 determined at the protein level: V_H Q1E, V_L V104L and T109A (variants are denoted by the amino acid residue and number followed by the replacement amino acid). The human γ1 constant regions are identical to those reported by Ellison et al. (Ellison, J. W. et al., *Nucleic Acids Res.* 13:4071-4079 (1982))

30   except for the mutations E359D and M361L (Eu numbering, as in Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987)) which we installed to convert the antibody from the naturally rare A allotype to the much more common non-A allotype (Tramontano, A. et al., *J. Mol. Biol.* 215:175-182 (1990)). This was an attempt to reduce the risk of anti-allotype antibodies interfering with

35   therapy.

Construction of Humanized Genes. Genes encoding chMAb4D5 light chain and heavy chain Fd fragment (V_H and C_H1 domains) were subcloned together into pUC119 (Vieira, J. & Messing, J., *Methods Enzymol.* 153:3-11 (1987)) to create pAK1 and simultaneously

humanized in a single step (Fig. 2). Briefly, sets of 6 contiguous oligonucleotides were designed to humanize V_H and V_L (Fig. 1). These oligonucleotides are 28 to 83 nucleotides in length, contain zero t 19 mismatches t the murine antibody templat and are c nstrained to have 8 or 9 perfectly matched residues at each end to promote efficient annealing and

5   ligation of adjacent oligonucleotides. The sets of V_H and V_L humanization oligonucleotides (5 pmol each) were phosphorylated with either ATP or γ-^{32}P-ATP (Carter, P. *Methods Enzymol.* 154:382-403 (1987)) and separately annealed with 3.7 pmol of pAK1 template in 40 μl 10 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$ by cooling from 100 ^{o}C to room temperature over $-$30 min. The annealed oligonucleotides were joined by incubation with T4

10   DNA ligase (12 units; New England Biolabs) in the presence of 2 μl 5 mM ATP and 2 μl 0.1 M DTT for 10 min at 14 ^{o}C. After electrophoresis on a 6% acrylamide sequencing gel the assembled oligonucleotides were located by autoradiography and recovered by electroelution. The assembled oligonucleotides ($-$0.3 pmol each) were simultaneously annealed to 0.15 pmol single-stranded deoxyuridine-containing pAK1 prepared according to Kunkel et al. (Kunkel, T.

15   A. *et al., Methods Enzymol.* 154:367-382 (1987)) in 10 μl 40 mM Tris-HCl (pH 7.5) and 16 mM $MgCl_2$ as above. Heteroduplex DNA was constructed by extending the primers with T7 DNA polymerase and transformed into *E. coli* BMH 71-18 *mutL* as previously described (Carter, P., in *Mutagenesis: A Practical Approach*, Chapter 1 (IRL Press, Oxford, UK 1991)). The resultant phagemid DNA pool was enriched first for huV_L by restriction purification using

20   *Xho*I and then for huV_H by restriction selection using *Stu*I as described in Carter, P., in *Mutagenesis: A Practical Approach*, Chapter 1 (IRL Press, Oxford, UK 1991); and in Wells, J. A. *et al., Phil. Trans. R. Soc. Lond. A* 317:415-423 (1986). Resultant clones containing both huV_L and huV_H genes were identified by nucleotide sequencing (Sanger, F. *et al., Proc. Natl. Acad. Sci. USA* 74:5463-5467 (1977)) and designated pAK2. Additional humanized

25   variants were generated by site-directed mutagenesis (Carter, P., in *Mutagenesis: A Practical Approach*, Chapter 1 (IRL Press, Oxford, UK 1991)). The muMAb4D5 V_L and V_H gene segments in the transient expression vectors described above were then precisely replaced with their humanized versions.

Expression and Purification of MAb4D5 Variants. Appropriate MAb4D5 light and heavy

30   chain cDNA expression vectors were co-transfected into an adenovirus transformed human embryonic kidney cell line, 293 (Graham, F. L. *et al., J. Gen. Virol.* 36:59-72 (1977)) using a high efficiency procedure (Gorman, C. M. *et al., DNA & Prot. Engin. Tech.* 2:3-10 (1990); Gorman, C., in *DNA Cloning*, vol II, pp 143-190 (D. M. Glover, ed., IRL Press, Oxford, UK 1985)). Media were harvested daily for up to 5 days and the cells re-fed with serum free

35   media. Antibodies were recovered from the media and affinity purified on protein A sepharose CL-4B (Pharmacia) as described by the manufacturer. The eluted antibody was buffer-exchanged into phosphate-buffered saline by G25 gel filtration, concentrated by ultrafiltration (Centriprep-30 r Centricon-100, Amicon), sterile-filtered (Millex-GV, Millipore)

and st red at 4 °C. The concentration of antibody was determined by using both total immunoglobulin and antigen binding ELISAs. The standard used was huMAb4D5-5, whos concentrati n had been determined by amin acid mp ition analysis.

Cell Proliferation Assay. The effect of MAb4D5 variants upon proliferation of the human mammary adenocarcinoma cell line, SK-BR-3, was investigated as previously described (Fendly, B. M. et al., Cancer Res. 50:1550-1558 (1990)) using saturating MAb4D5 concentrations.

Affinity Measurements. The antigen binding affinity of MAb4D5 variants was determined using a secreted form of the p185^{HER2} ECD prepared as described in Fendly, B. M. et al., J. Biol. Resp. Mod. 9:449-455 (1990). Briefly, antibody and p185^{HER2} ECD were incubated in solution until equilibrium was found to be reached. The concentration of free antibody was then determined by ELISA using immobilized p185^{HER2} ECD and used to calculate affinity (K_d) according to Friguet et al. (Friguet, B. et al., J. Immunol. Methods 77:305-319 (1985)).

RESULTS

Humanization of muMAb4D5. The muMAb4D5 V_L and V_H gene segments were first cloned by PCR and sequenced (Fig. 1). The variable genes were then simultaneously humanized by gene conversion mutagenesis using preassembled oligonucleotides (Fig. 2). A 311-mer oligonucleotide containing 39 mismatches to the template directed 24 simultaneous amino acid changes required to humanize muMAb4D5 V_L. Humanization of muMAb4D5 V_H required 32 amino acid changes which were installed with a 361-mer containing 59 mismatches to the muMAb4D5 template. Two out of 8 clones sequenced precisely encode huMAb4D5-5, although one of these clones contained a single nucleotide imperfection. The 6 other clones were essentially humanized but contained a small number of errors: < 3 nucleotide changes and < 1 single nucleotide deletion per kilobase. Additional humanized variants (Table 3) were constructed by site-directed mutagenesis of huMAb4D5-5.

Expression levels of huMAb4D5 variants were in the range of 7 to 15 μg/ml as judged by ELISA using immobilized p185^{HER2} ECD. Successive harvests of five 10 cm plates allowed 200 μg to 500 mg of each variant to be produced in a week. Antibodies affinity purified on protein A gave a single band on a Coomassie blue stained SDS polyacrylamide gel of mobility consistent with the expected M_r of ~150 kDa. Electrophoresis under reducing conditions gave 2 bands consistent with the expected M_r of free heavy (48 kDa) and light (23 kDa) chains (not shown). Amino terminal sequence analysis (10-cycles) gave the mixed sequence expected (see Fig. 1) from an equimolar combination of light and heavy chains (not shown).

huMAb4D5 Variants. In general, the FR residues were chosen from consensus human sequences (Kabat, E. A. et al., Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, MD, 1987)) and CDR residues from muMAb4D5. Additional

-59-

variants wer constructed by replacing selected human r sidues in huMAb4D5-1 with their muMAb4D5 ounterparts. These are V_H residues 71, 73, 78, 93 plu 102 and V_L residues 55 plus 66 identified by our m lecular modeling. V_H residue 71 has pr viously been proposed by others (Tramontano, A. et al., J. Mol. Biol. 215:175-182 (1990)) to be critical to the

5 conformation of V_H-CDR2. Amino acid sequence differences between huMAb4D5 variant molecules are shown in Table 3, together with their $p185^{HER2}$ ECD binding affinity and maximal anti-proliferative activities against SK-BR-3 cells. Very similar K_d values were obtained for binding of MAb4D5 variants to either SK-BR-3 cells or to $p185^{HER2}$ ECD (Table 3). However, K_d estimates derived from binding of MAb4D5 variants to $p185^{HER2}$ ECD were

10 more reproducible with smaller standard errors and consumed much smaller quantities of antibody than binding measurements with whole cells.

The most potent humanized variant designed by molecular modeling, huMAb4D5-8, contains 5 FR residues from muMAb4D5. This antibody binds the $p185^{HER2}$ ECD 3-fold more tightly than does muMAb4D5 itself (Table 3) and has comparable anti-proliferative activity

15 with SK-BR-3 cells (Fig. 3). In contrast, huMAb4D5-1 is the most humanized but least potent muMAb4D5 variant, created by simply installing the muMAb4D5 CDRs into the consensus human sequences. huMAb4D5-1 binds the $p185^{HER2}$ ECD 80-fold less tightly than does the murine antibody and has no detectable anti-proliferative activity at the highest antibody concentration investigated (16 μg/ml).

20 The anti-proliferative activity of huMAb4D5 variants against $p185^{HER2}$ overexpressing SK-BR-3 cells is not simply correlated with their binding affinity for the $p185^{HER2}$ ECD. For example, installation of three murine residues into the V_H domain of huMAb4D5-2 (D73T, L78A and A93S) to create huMAb4D5-3 does not change the antigen binding affinity but does confer significant anti-proliferative activity (Table 3).

25 The importance of V_H residue 71 (Tramontano, A. et al., J. Mol. Biol. 215:175-182 (1990)) is supported by the observed 5-fold increase in affinity for $p185^{HER2}$ ECD on replacement of R71 in huMAb4D5-1 with the corresponding murine residue, alanine (huMAb4D5-2). In contrast, replacing V_H L78 in huMAb4D5-4 with the murine residue, alanine (huMAb4D5-5), does not significantly change the affinity for the $p185^{HER2}$ ECD or

30 change anti-proliferative activity, suggesting that residue 78 is not of critical functional significance to huMAb4D5 and its ability to interact properly with the extracellular domain of $p185^{HER2}$.

V_L residue 66 is usually a glycine in human and murine κ chain sequences (Kabat, E. A. et al., Sequences of Proteins of Immunological Interest (National Institutes of Health,

35 Bethesda, MD, 1987)) but an arginine occupies this position in the muMAb4D5 k light chain. The side chain of residue 66 is likely to affect the conformation of V_L-CDR1 and V_L-CDR2 and the hairpin turn at 68-69 (Fig. 4). Consistent with the importance of this residue, the

mutation V_L G66R (huMAb4D5-3 → huMAb4D5-5) increas s the affinity for the p185^{HER2} ECD by 4-f ld with a concomitant increase in anti-proliferative activity.

From m lecular modeling it appears that the tyrosyl side chain of muMAb4D5 V_L residue 55 may either stabilize the conformation of V_H-CDR3 or provide an interaction at the

5   V_L-V_H interface. The latter function may be dependent upon the presence of V_H Y102. In the context of huMAb4D5-5 the mutations V_L E55Y (huMAb4D5-6) and V_H V102Y (huMAb4D5-7) individually increase the affinity for p185^{HER2} ECD by 5-fold and 2-fold respectively, whereas together (huMAb4D5-8) they increase the affinity by 11-fold. This is consistent with either proposed role of V_L Y55 and V_H Y102.

10   Secondary Immuno Function of huMAb4D5-8. MuMAb4D5 inhibits the growth of human breast tumor cells which overexpress p185^{HER2} (Hudziak, R. M. et al., Molec. Cell. Biol. 9:1165-1172 (1989)). The antibody, however, does not offer the possibility of direct tumor cytotoxic effects. This possibility does arise in huMAb4D5-8 as a result of its high affinity (K_d = 0.1 µM) and its human IgG$_1$ subtype. Table 4 compares the ADCC mediated

15   by huMAb4D5-8 with muMAb4D5 on a normal lung epithelial cell line, WI-38, which expresses a low level of p185^{HER2} and on SK-BR-3, which expresses a high level of p185^{HER2}. The results demonstrate that: (1) huMAb4D5 has a greatly enhanced ability to carry out ADCC as compared with its murine parent; and (2) that this activity may be selective for cell types which overexpress p185^{HER2}.

20

DISCUSSION

MuMAb4D5 is potentially useful for human therapy since it is cytostatic towards human breast and ovarian tumor lines overexpressing the HER2-encoded p185^{HER2} receptor-like tyrosine kinase. Since both breast and ovarian carcinomas are chronic diseases

25   it is anticipated that the optimal MAb4D5 variant molecule for therapy will have low immunogenicity and will be cytotoxic rather than solely cytostatic in effect. Humanization of muMAb4D5 should accomplish these goals. We have identified 5 different huMAb4D5 variants which bind tightly to p185^{HER2} ECD (K_d ≤ 1 nM) and which have significant anti-proliferative activity (Table 3). Furthermore huMAb4D5-8 but not muMAb4D5 mediates

30   ADCC against human tumor cell lines overexpressing p185^{HER2} in the presence of human effector cells (Table 4) as anticipated for a human γ1 isotype (Brüggemann, M. et al., J. Exp. Med. 166:1351-1361 (1987); Riechmann, L. et al., Nature 332:323-327 (1988)).

Rapid humanization of huMAb4D5 was facilitated by the gene conversion mutagenesis strategy developed here using long preassembled oligonucleotides. This method requires less

35   than half the amount of synthetic DNA as does total gene synthesis and does not require convenient restriction sites in the target DNA. Our method appears to be simpler and more reliable than a variant protocol recently reported (Rostapshov, V. M. et al., FEBS Lett. 249:379-382 (1989)). Transient expression of huMAb4D5 in human embryonic kidney 293

cells permitted the is lation of a few hundred micr grams of huMAb4D5 variants f r rapid characterization by growth inhibition and antigen binding affinity assays. Furthermore, diff rent c mbinations of light and heavy chain were readily tested by co-transfection of corresponding cDNA expression vectors.

5   The crucial role of molecular modeling in the humanization of muMAb4D5 is illustrated by the designed variant huMAb4D5-8 which binds the p185^{HER2} ECD 250-fold more tightly than the simple CDR loop swap variant, huMAb4D5-1. It has previously been shown that the antigen binding affinity of a humanized antibody can be increased by mutagenesis based upon molecular modelling (Riechmann, L. et al., Nature 332:323-327 (1988); Queen, C. et al., Proc.

10   Natl. Acad. Sci. USA 86:10029-10033 (1989)). Here we have extended this earlier work by others with a designed humanized antibody which binds its antigen 3-fold more tightly than the parent rodent antibody. While this result is gratifying, assessment of the success of the molecular modeling must await the outcome of X-ray structure determination. From analysis of huMAb4D5 variants (Table 3) it is apparent that their anti-proliferative activity is not a

15   simple function of their binding affinity for p185^{HER2} ECD. For example the huMAb4D5-8 variant binds p185^{HER2} 3-fold more tightly than muMAb4D5 but the humanized variant is slightly less potent in blocking the proliferation of SK-BR-3 cells. Additional huMAb4D5 variants are currently being constructed in an attempt to identify residues triggering the anti-proliferative activity and in an attempt to enhance this activity.

20   In addition to retaining tight receptor binding and the ability to inhibit cell growth, the huMAb4D5-8 also confers a secondary immune function (ADCC). This allows for direct cytotoxic activity of the humanized molecule in the presence of human effector cells. The apparent selectivity of the cytotoxic activity for cell types which overexpress p185^{HER2} allows for the evolution of a straightforward clinic approach to those human cancers

25   characterized by overexpression of the HER2 protooncogene.

Table 3

p185^{HER2} ECD binding affinity and anti-pr liferative activities of MAb4D5 variants

MAb4D5 Relative cell Variant proliferation[‡]	V$_H$ Residue[°]					V$_L$ Residue[°]		K_d[†]	
	71	73	78	93	102	55	66		
	FR3	FR3	FR3	FR3	CDR3	CDR2	FR3	nM	
huMAb4D5-1	R	D	L	A	V	E	G	25	102
huMAb4D5-2	Ala	D	L	A	V	E	G	4.7	101
huMAb4D5-3	Ala	Thr	Ala	Ser	V	E	G	4.4	66
huMAb4D5-4	Ala	Thr	L	Ser	V	E	Arg	0.82	56
huMAb4D5-5	Ala	Thr	Ala	Ser	V	E	Arg	1.1	48
huMAb4D5-6	Ala	Thr	Ala	Ser	V	Tyr	Arg	0.22	51
huMAb4D5-7	Ala	Thr	Ala	Ser	Tyr	E	Arg	0.62	53
huMAb4D5-8	Ala	Thr	Ala	Ser	Tyr	Tyr	Arg	0.10	54
muMAb4D5	Ala	Thr	Ala	Ser	Tyr	Tyr	Arg	0.30	37

[°] Human and murine residues are shown in one letter and three letter amino acid code respectively.

[†] K_d values for the p185^{HER2} ECD were determined using the method of Friguet et al. (43) and the standard error of each estimate is ≤ ± 10%.

[‡] Proliferation of SK-BR-3 cells incubated for 96 hr with MAb4D5 variants shown as a percentage of the untreated control as described (Hudziak, R. M. et al., Molec. Cell. Biol. 9:1165-1172 (1989)). Data represent the maximal anti-proliferative effect for each variant (see Fig. 3A) calculated as the mean of triplicate determinations at a MAb4D5 concentration of 8 μg/ml. Data are all taken from the same experiment with an estimated standard error of ≤ ± 15%.

Table 4

Selectivity of antibody dependent tumor cell cytotoxicity mediated by huMAb4D5-8

	Effector:Target ratio[†]	WI-38[°]		SK-BR-3	
		muMAb4D5	huMAb4D5-8	muMAb4D5	huMAb4D5-8
A.[§]	25:1	<1.0	9.3	7.5	40.6
	12.5:1	<1.0	11.1	4.7	36.8
	6.25:1	<1.0	8.9	0.9	35.2
	3.13:1	<1.0	8.5	4.6	19.6
B.	25:1	<1.0	3.1	6.1	33.4
	12.5:1	<1.0	1.7	5.5	26.2
	6.25:1	1.3	2.2	2.0	21.0
	3.13:1	<1.0	0.8	2.4	13.4

[°] Sensitivity to ADCC of two human cell lines (WI-38, normal lung epithelium; and SK-BR-3, human breast tumor cell line) are compared. WI-38 expresses a low level of p185[HER2] (0.6 pg per μg cell protein) and SK-BR-3 expresses a high level of p185[HER2] (64 pg p185[HER2] per μg cell protein), as determined by ELISA (Fendly et al., J. Biol. Resp. Mod. 9:449-455 (1990)).

[†] ADCC assays were carried out as described in Brüggemann et al., J. Exp. Med. 166:1351-1361 (1987). Effector to target ratios were of IL-2 activated human peripheral blood lymphocytes to either WI-38 fibroblasts or SK-BR-3 tumor cells in 96-well microtiter plates for 4 hours at 37 °C. Values given represent percent specific cell lysis as determined by ^{51}Cr release. Estimated standard error in these quadruplicate determinations was ≤ ±10%.

[§] Monoclonal antibody concentrations used were 0.1 μg/ml (A) and 0.1 μg/ml (B).

EXAMPLE 2. Schematic Meth d for Humanizing an Antibody Sequence

This example illustrates one stepwise laboration of the methods for creating a humanized sequence described above. It will be understood that not all of these steps are essential to the claimed invention, and that steps may be taken in different order.

5   1.   ascertain a consensus human variable domain amino acid sequence and prepare from it a consensus structural model.

       2.   prepare model of import (the non-human domain to be humanized) variable domain sequences and note structural differences with respect to consensus human model.

       3.   identify CDR sequences in human and in import, both by using Kabat (supra, 1987) and
10   crystal structure criteria. If there is any difference in CDR identity from the different criteria, use of crystal structure definition of the CDR, but retain the Kabat residues as important framework residues to import.

       4.   substitute import CDR sequences for human CDR sequences to obtain initial "humanized" sequence.

15   5.   compare import non-CDR variable domain sequence to the humanized sequence and note divergences.

       6.   Proceed through the following analysis for each amino acid residue where the import diverges from the humanized.

             a.   If the humanized residue represents a residue which is generally highly conserved
20   across all species, use the residue in the humanized sequence. If the residue is not conserved across all species, proceed with the analysis described in 6b.

             b.   If the residue is not generally conserved across all species, ask if the residue is generally conserved in humans.

                   i.   If the residue is generally conserved in humans but the import residue
25   differs, examine the structural models of the import and human sequences and determine if the import residue would be likely to affect the binding or biological activity of the CDRs by considering 1) could it bind antigen directly and 2) could it affect the conformation of the CDR. If the conclusion is that an affect on the CDRs is likely, substitute the import
30   residue. If the conclusion is that a CDR affect is unlikely, leave the humanized residue unchanged.

                   ii.   If the residue is also not generally conserved in humans, examine the structural models of the import and human sequences and determine if the import residue would be likely to affect the binding or biological activity
35   of the CDRs be considering 1) could it bind antigen directly and 2) could it affect the conformation of the CDR. If the conclusion is that an affect on the CDRs is likely, substitute the import residue. If the conclusion is that a CDR affect is unlikely, proceed to the next step.

-65-

a)   Examine the structural models of the import and human sequences
         and determine if the residue is exposed on the surface f the
         domain or is buried within. If the residue is expos d, use the
         residue in the humanized sequence. If the residue is buried,
5   proceed to the next step.

         (i)   Examine the structural models of the import and human
                 sequences and determine if the residue is likely to affect the
                 V_L - V_H interface. Residues involved with the interface
                 include: 34L, 36L, 38L, 43L, 33L, 36L, 85L, 87L, 89L, 91L,
10   96L, 98L, 35H, 37H, 39H, 43H, 45H, 47H, 60H, 91H, 93H,
                 95H, 100H, and 103H. If no effect is likely, use the residue
                 in the humanized sequence. If some effect is likely,
                 substitute the import residue.

7.   Search the import sequence, the consensus sequence and the humanized sequence for
15   glycosylation sites outside the CDRs, and determine if this glycosylation site is likely
         to have any affect on antigen binding and/or biological activity. If no effect is likely,
         use the human sequence at that site; if some affect is likely, eliminate the glycosylation
         site or use the import sequence at that site.

8.   After completing the above analysis, determine the planned humanized sequence and
20   prepare and test a sample. If the sample does not bind well to the target antigen,
         examine the particular residues listed below, regardless of the question of residue
         identity between the import and humanized residues.

         a.   Examine particular peripheral (non-CDR) variable domain residues that may, due
                 to their position, possibly interact directly with a macromolecular antigen,
25   including the following residues (where the ° indicates residues which have been
                 found to interact with antigen based on crystal structures):

                 i.   Variable light domain: 36, 46, 49°, 63-70
                 ii.   Variable heavy domain: 2, 47°, 68, 70, 73-76.

         b.   Examine particular variable domain residues which could interact with, or
30   otherwise affect, the conformation of variable domain CDRs, including the
                 following (not including CDR residues themselves, since it is assumed that,
                 because the CDRs interact with one another, any residue in one CDR could
                 potentially affect the conformation of another CDR residue) (L = LIGHT,
                 H = HEAVY, residues appearing in bold are indicated to be structurally important
35   according the Chothia et al., Nature 342:877 (1989), and residues appearing in
                 italic were altered during humanization by Queen et al. (PDL), Proc. Natl. Acad.
                 Sci. USA 86:10029 (1989) and Proc. Natl. Acad. Sci. USA 88:2869 (1991).):
                 i.   Variable light domain:

   a)   CDR-1 (r sidues 24L-34L): 2L, 4L, 66L-69L, 71L

   b)   CDR-2 (r sidues 50L-56L): 35L, 46L, *47L*, 48L, *49L*, 58L, 62L,
        64L-66L, 71L, 73L

   c)   CDR-3 (residues 89L-97L): 2L, 4L, 36L, 98L, 37H, 45H, 47H,
        58H, 60H

ii.   Variable heavy domain:

   a)   CDR-1 (residues 26H-35H): 2H, 4H, 24H, 36H, 71H, 73H, 76H,
        78H, 92H, ~~94H~~

   b)   CDR-2 (residues 50H-65H): 49H, 69H, 69H, 71H, 73H, 78H

   c)   CDR-3 (residues 95H-102H): examine all residues as possible
        interaction partners with this loop, because this loop varies in size
        and conformation much more than the other CDRs.

9.   If after step 8 the humanized variable domain still is lacking in desired binding, repeat
     step 8. In addition, re-investigate any buried residues which might affect the V_L - V_H
     interface (but which would not directly affect CDR conformation). Additionally,
     evaluate the accessibility of non-CDR residues to solvent.

EXAMPLE 3. Engineering a Humanized Bispecific F(ab')₂ Fragment

     This example demonstrates the construction of a humanized bispecific antibody
(BsF(ab')₂v1 by separate *E. coli* expression of each Fab' arm followed by directed chemical
coupling *in vitro*. BsF(ab')₂ v1 (anti-CD3 / anti-p185[HER2]) was demonstrated to retarget the
cytotoxic activity of human CD3⁺ CTL *in vitro* against the human breast tumor cell line, SK-
BR-3, which overexpresses the p185[HER2] product of the protooncogene *HER2*. This example
demonstrates the minimalistic humanization strategy of installing as few murine residues as
possible into a human antibody in order to recruit antigen-binding affinity and biological
properties comparable to that of the murine parent antibody. This strategy proved very
successful for the anti-p185[HER2]arm of BsF(ab')₂v1. In contrast BsF(ab')₂ v1 binds to T cells
via its anti-CD3 arm much less efficiently than does the chimeric BsF(ab')₂ which contains the
variable domains of the murine parent anti-CD3 antibody. Here we have constructed
additional BsF(ab')₂ fragments containing variant anti-CD3 arms with selected murine residues
restored in an attempt to improve antibody binding to T cells. One such variant, Bs F(ab')₂
v9, was created by replacing six residues in the second hypervariable loop of the anti-CD3
heavy chain variable domain of BsF(ab')₂ v1 with their counterparts from the murine parent
anti-CD3 antibody. BsF(ab')₂ v9 binds to T cells (Jurkat) much more efficiently than does
BsF(ab')₂ v1 and almost as efficiently as the chimeric BsF(ab')₂. This improvement in the
efficiency of T cell binding of the humanized BsF(ab')₂ is an important step in its development
as a potential therapeutic agent for the treatment of p185[HER2]-overexpressing cancers.

Bispecific antibodies (BsAbs) with specificities for tumor-associated antigens and surface markers n immune effector cells have proved effectiv for retargeting effector cells t kill tumor targets b th *in vitro* and *in vivo* (reviewed by Fanger, M. W. *et al.*, *Immunol. Today* 10: 92-99 (1989); Fanger, M. W. *et al.*, *Immunol. Today* 12: 51-54 (1991); and

5   Nelson, H., *Cancer Cells* 3: 163-172 (1991)). BsF(ab')$_2$ fragments have often been used In preference to intact BsAbs in retargeted cellular cytotoxicity to avoid the risk of killing innocent bystander cells binding to the Fc region of the antibody. An additional advantage of BsF(ab')$_2$ over intact BsAbs is that they are generally much simpler to prepare free of contaminating monospecific molecules (reviewed by Songsivilai, S. and Lachmann, P. J., *Clin.*

10   *Exp. Immunol.* 79: 315-321 (1990) and Nolan, O. and O'Kennedy, R., *Biochim. Biophys. Acta* 1040: 1-11 (1990)).

BsF(ab')$_2$ fragments are traditionally constructed by directed chemical coupling of Fab' fragments obtained by limited proteolysis plus mild reduction of the parent rodent monoclonal Ab (Brennan, M. *et al.*, *Science* 229, 81-83 (1985) and Glennie, M. J. *et al.*, *J. Immunol.* 139:

15   2367-2375 (1987)). One such BsF(ab')$_2$ fragment (anti-glioma associated antigen / anti-CD3) was found to have clinical efficacy in glioma patients (Nitta, T. *et al.*, *Lancet* 335: 368-371 (1990) and another BsF(ab')$_2$ (anti-indium chelate / anti-carcinoembryonic antigen) allowed clinical imaging of colorectal carcinoma (Stickney, D. R. *et al.*, *Antibody, Immunoconj. Radiopharm.* 2: 1-13 (1989)). Future BsF(ab')$_2$ destined for clinical applications are likely to

20   be constructed from antibodies which are either human or at least "humanized" (Riechmann, L. *et al.*, *Nature* 332: 323-327 (1988) to reduce their immunogenicity (Hale, G. *et al.*, *Lancet* I: 1394-1399 (1988)).

Recently a facile route to a fully humanized BsF(ab')$_2$ fragment designed for tumor immunotherapy has been demonstrated (Shalaby, M. R. *et al.*, *J. Exp. Med.* 175: 217-225

25   (1992)). This approach involves separate *E. coli* expression of each Fab' arm followed by traditional directed chemical coupling *in vitro* to form the BsF(ab')$_2$. One arm of the BsF(ab')$_2$ was a humanized version (Carter, P. *et al.*, *Proc. Natl. Acad. Sci. USA* (1992a) and Carter, P., *et al.*, *Bio/Technology* 10: 163-167 (1992b)) of the murine monoclonal Ab 4D5 which is directed against the p185^{HER2} product of the protooncogene *HER2* (c-*erbB*-2) (Fendly, B. M.

30   *et al.*, *Cancer Res.* 50: 1550-1558 (1989)). The humanization of the antibody 4D5 is shown In Example 1 of this application. The second arm was a minimalistically humanized anti-CD3 antibody (Shalaby *et al. supra*) which was created by installing the CDR loops from the variable domains of the murine parent monoclonal Ab UCHT1 (Beverley, P. C. L. and Callard, R. E., *Eur. J. Immunol.* 11: 329-334 (1981)) into the humanized anti-p185^{HER2} antibody. The

35   BsF(ab')$_2$ fragment containing the most potent humanized anti-CD3 variant (v1) was demonstrated by flow cytometry to bind specifically to a tumor target overexpressing p185^{HER2} and to human peripheral blood mononuclear cells carrying CD3. In addition, Bs F(ab')$_2$ v1 enhanced the cytotoxic effects of activated human CTL 4-fold against SK-BR-3 tumor cells

overexpressing p185^{HER2}. The example descries efforts to improve the antigen binding affinity of the humanized anti-CD3 arm by the judicious r cruitment of a small number of additional murine residu s into the minimalistically humanized anti-CD3 variable domains.

<div align="center">

MATERIALS AND METHODS

</div>

5   *Construction of mutations in the anti-CD3 variable region genes.* The construction of genes encoding humanized anti-CD3 variant 1 (v1) variable light (V$_L$) and heavy (V$_H$) chain domains in phagemid pUC119 has been described (Shalaby *et al. supra*). Additional anti-CD3 variants were generated using an efficient site-directed mutagenesis method (Carter, P.,

10   *Mutagenesis: a practical approach*, (M. J. McPherson, Ed.), Chapter 1, IRL Press, Oxford, UK (1991)) using mismatched oligonucleotides which either install or remove unique restriction sites. Oligonucleotides used are listed below using lowercase to indicate the targeted mutations. Corresponding coding changes are denoted by the starting amino acid in one letter code followed by the residue numbered according to Kabat, E. A. *et al., Sequences of Proteins*

15   *of Immunological Interest*, 5th edition, National Institutes of Health, Bethesda, MD, USA (1991), then the replacement amino acid and finally the identity of the anti-CD3 variant:

         HX11, 5' GTAGATAAATCCtctAACACAGCCTAtCTGCAAATG 3' (SEQ.ID. NO. 11) V$_H$ K75S, v6;

         HX12, 5' GTAGATAAATCCAAAtctACAGCCTAtCTGCAAATG 3' (SEQ.ID. NO. 12) V$_H$

20   N76S, v7;

         HX13, 5' GTAGATAAATCCtcttctACAGCCTAtCTGCAAATG 3' (SEQ.ID. NO. 13) V$_H$ K75S:N76S, v8;

         X14, 5' CTTATAAAGGTGTTtCcACCTATaaCcAgAaatTCAA GGatCGTTTCACgATAtcCGTAGATAAATCC 3' (SEQ.ID.NO. 14)

25   V$_H$ T57S:A60N:D61Q:S62K:V63F:G65D, v9;

         LX6, 5' CTATACCTCCCGTCTgcatTCTGGAGTCCC 3' (SEQ.ID. NO. 15) V$_L$ E55H, v11.

Oligonucleotides HX11, HX12 and HX13 each remove a site for BspMI, whereas LX6 removes a site for XhoI and HX14 installs a site for EcoRV (bold). Anti-CD3 variant v10 was

30   constructed from v9 by site-directed mutagenesis using oligonucleotide HX13. Mutants were verified by dideoxynucleotide sequencing (Sanger, F. *et al., Proc. Natl. Acad. Sci. USA* 74: 5463-5467 (1977)).

         E. coli expression of Fab' fragments. The expression plasmid, pAK19, for the co-secretion of light chain and heavy chain Fd' fragment of the most preferred humanized anti-

35   p185^{HER2} variant, HuMAb4D5-8, is described in Carter *et al.,* 1992b, *supra.* Briefly, the Fab' expression unit is bicistronic with both chains under the transcriptional control of the *phoA* promoter. Genes encoding humanized V$_L$ and V$_H$ domains are precisely fused on their 5' side to a gene segment encoding the heat-stable enterotoxin II signal sequence and on their 3' side

<div align="center">

-69-

</div>

to human k, C_L and IgG1 C_H1 constant domain genes, respectively. The C_H1 gene is immediately followed by a sequence encoding the hinge sequence CysAlaAla and followed by a bacteriophage λ t_0 transcriptional terminat r. Fab' expression plasmids for chimeric and humaniz d anti-CD3 variants (v1 t v4, Shalaby et al.,supra; v6 to v12, this study) were

5 created from pAK19 by precisely replacing anti-p185^{HER2} V_L and V_H gene segments with those encoding murine and corresponding humanized variants of the anti-CD3 antibody, respectively, by sub-cloning and site-directed mutagenesis. The Fab' expression plasmid for the most potent humanized anti-CD3 variant identified in this study (v9) is designated pAK22. The anti-p185^{HER2} Fab' fragment was secreted from E. coli K12 strain 25F2 containing plasmid

10 pAK19 grown for 32 to 40 hr at 37°C in an aerated 10 liter fermentor. The final cell density was 120-150 OD$_{050}$ and the titer of soluble and functional anti-p185^{HER2} Fab' was 1-2 g/liter as judged by antigen binding ELISA (Carter et al., 1992b, supra). Anti-CD3 Fab' variants were secreted from E. coli containing corresponding expression plasmids using very similar fermentation protocols. The highest expression titers of chimeric and humanized anti-CD3

15 variants were 200 mg/liter and 700 mg/liter, respectively, as judged by total immunoglobulin ELISA.

      Construction of BsF(ab')₂ fragments. Fab' fragments were directly recovered from E. coli fermentation pastes in the free thiol form (Fab'-SH) by affinity purification on Streptococcal protein G at pH 5 in the presence of EDTA (Carter et al., 1992b supra).

20 Thioether linked BsF(ab')₂ fragments (anti-p185^{HER2} / anti-CD3) were constructed by the procedure of Glennie et al. supra with the following modifications. Anti-p185^{HER2} Fab'-SH in 100 mM Tris acetate, 5 mM EDTA (pH 5.0) was reacted with 0.1 vol of 40 mM N,N'-1,2-phenylenedimalemide (o-PDM) in dimethyl formamide for ~1.5 hr at 20°C. Excess o-PDM was removed by protein G purification of the Fab' maleimide derivative (Fab'-mal) followed by

25 buffer exchange into 20 mM sodium acetate, 5 mM EDTA (pH 5.3) (coupling buffer) using centriprep-30 concentrators (Amicon). The total concentration of Fab' variants was estimated from the measured absorbance at 280 nm (HuMAb4D5-8 Fab' $e^{0.1\%}$ = 1.56, Carter et al., 1992b, supra). The free thiol content of Fab' preparations was estimated by reaction with 5, 5'-dithiobis(2-nitrobenzoic acid) as described by Creighton, T. E., *Protein structure: a*

30 *practical approach*, (T. E. Creighton, Ed.), Chapter 7, IRL Press, Oxford, UK (1990). Equimolar amounts of anti-p185^{HER2} Fab'-mal (assuming quantitative reaction of Fab'-SH with o-PDM) and each anti-CD3 Fab'-SH variant were coupled together at a combined concentration of 1 to 2.5 mg/ml in the coupling buffer for 14 to 48 hr at 4°C. The coupling reaction was adjusted to 4 mM cysteine at pH 7.0 and incubated for 15 min at 20°C to reduce any unwanted

35 disulfide-linked F(ab')₂ formed. These reduction conditions are sufficient to reduce inter-heavy chain disulfide bonds with virtually no reduction of the disulfide between light and heavy chains. Any free thiols generated were then blocked with 50 mM iodoacetamide. BsF(ab')₂ was isolated from the coupling reaction by S100-HR (Pharmacia) size exclusion

chromat graphy (2.5 cm x 100 cm) in th presence of PBS. The BsF(ab')₂ samples were
passed through a 0.2 mm filter flash froz n in liquid nitrogen and stored at -70°C.

    Flow cytometric analysis of F(ab')₂ binding to Jurkat cells. Th Jurkat human acute
T cell leukemia cell line was purchased from the American Type Culture Collection (Rockville,
5   MD) (ATCC TIB 152) and grown as recommended by the ATCC. Aliquots of 10⁶ Jurkat cells
were incubated with appropriate concentrations of BsF(ab')₂ (anti-p185^{KER2} / anti-CD3 variant)
or control mono-specific anti-p185^{KER2} F(ab')₂ in PBS plus 0.1% (w/v) bovine serum albumin
and 10 mM sodium azide for 45 min at 4 °C. The cells were washed and then incubated with
fluorescein-conjugated goat anti-human F(ab')₂ (Organon Teknika, West Chester, PA) for 45
10   min at 4 °C. Cells were washed and analyzed on a FACScan° (Becton Dickinson and Co.,
Mountain View, CA). Cells (8 x 10³) were acquired by list mode and gated by forward light
scatter *versus* side light scatter excluding dead cells and debris.

RESULTS

15   *Design of humanized anti-CD3 variants.* The most potent humanized anti-CD3 variant
previously identified, v1, differs from the murine parent antibody, UCHT1 at 19 out of 107
amino acid residues within V_L and at 37 out of 122 positions within V_H (Shalaby *et al.,supra*)
1992). Here we recruited back additional murine residues into anti-CD3 v1 in an attempt to
improve the binding affinity for CD3. The strategy chosen was a compromise between
20   minimizing both the number of additional murine residues recruited and the number of anti-
CD3 variants to be analyzed. We focused our attentions on a few CDR residues which were
originally kept as human sequences in our minimalistic humanization regime. Thus human
residues in V_H CDR2 of anti-CD3 v1 were replaced *en bloc* with their murine counterparts to
give anti-CD3 v9: T57S:A60N:D61Q:S62K:V63F:G65D (Fig. 5). Similarly, the human residue
25   E55 in V_L CDR2 of anti-CD3 v1 was replaced with histidine from the murine anti-CD3 antibody
to generate anti-CD3 v11. In addition, V_H framework region (FR) residues 75 and 76 in anti-
CD3 v1 were also replaced with their murine counterparts to create anti-CD3 v8: K75S:N76S.
V_H residues 75 and 76 are located in a loop close to V_H CDR1 and CDR2 and therefore might
influence antigen binding. Additional variants created by combining mutations at these three
30   sites are described below.

    Preparation of BsF(ab')₂ fragments. Soluble and functional anti-p185^{HER2} and anti-CD3
Fab' fragments were recovered directly from corresponding *E. coli* fermentation pastes with
the single hinge cysteine predominantly in the free thiol form (75-100 % Fab'-SH) by affinity
purification on Streptococcal protein G at pH 5 in the presence of EDTA (Carter *et al.*, 1992b,
35   *supra*). Thioether-linked BsF(ab')₂ fragments were then constructed by directed coupling using
o-PDM as described by Glennie *et al., supra*. One arm was always the most potent humanized
anti-p185^{HER2} variant, HuMAb4D5-8 (Carter *et al.*, 1992a, *supra*) and the other either a
chimeric or humanized variant of the anti-CD3 antibody. Anti-p185^{KER2} Fab'-SH was reacted

with o-PDM to f rm th maleimid derivative (Fab'-mal) and then c upled to the Fab'-SH f r

ach anti-CD3 variant. F(ab')₂ was then purified away fr m unreacted Fab' by siz exclusion

chromatography as shown for a representative preparati n (BsF(ab')₂ v8) in data not shown.

The F(ab')₂ fragment represents ~54% of the total amount of antibody fragments (by mass) as

5   judged by integration of the chromatograph peaks.

SDS-PAGE analysis of this BsF(ab')₂ v8 preparation under non-reducing conditions gave

one major band with the expected mobility (M_r ~ 96 kD) as well as several very minor bands

(data not shown). Amino-terminal sequence analysis of the major band after electroblotting

on to polyvinylidene difluoride membrane Matsudaira, P., J. Biol. Chem. 262: 10035-10038

10   (1987) gave the expected mixed sequence from a stoichiometric 1:1 mixture of light and

heavy chains (V_L / V_H: D/E, I/V, Q/Q, M/L, T/V, Q/E, S/S) expected for BsF(ab')₂. The amino

terminal region of both light chains are identical as are both heavy chains and correspond to

consensus human FR sequences. We have previously demonstrated that F(ab')₂ constructed

by directed chemical coupling carry both anti-p185[HER2] and anti-CD3 antigen specificities

15   (Shalaby et al., supra). The level of contamination of the BsF(ab')₂ with monospecific F(ab'

)₂ is likely to be very low since mock coupling reactions with either anti-p185[HER2] Fab'-mal or

anti-CD3 Fab'-SH alone did not yield detectable quantities of F(ab')₂. Furthermore the

coupling reaction was subjected to a mild reduction step followed by alkylation to remove

trace amounts of disulfide-linked F(ab')₂ that might be present. SDS-PAGE of the purified

20   F(ab')₂ under reducing conditions gave two major bands with electrophoretic mobility and

amino terminal sequence anticipated for free light chain and thioether-linked heavy chain

dimers.

Scanning LASER densitometry of a o-PDM coupled F(ab')₂ preparation suggest that the

minor species together represent ~10% of the protein. These minor contaminants were

25   characterized by amino terminal sequence analysis and were tentatively identified on the basis

of stoichiometry of light and heavy chain sequences and their electrophoretic mobility (data

not shown). These data are consistent with the minor contaminants including imperfect F(ab'

)₂ in which the disulfide bond between light and heavy chains is missing in one or both arms,

trace amounts of Fab' and heavy chain thioether-linked to light chain.

30   *Binding of BsF(ab')₂ to Jurkat cells.* Binding of BsF(ab')₂ containing different anti-CD3

variants to Jurkat cells (human acute T cell leukemia) was investigated by flow cytometry

(data not shown). BsF(ab')₂ v9 binds much more efficiently to Jurkat cells than does our

starting molecule, BsF(ab')₂ v1, and almost as efficiently as the chimeric BsF(ab')₂. Installation

of additional murine residues into anti-CD3 v9 to create v10 (V_H K75S:N76S) and v12 (V_H

35   K75S:N76S plus V_L E55H) did not further improve binding of corresponding BsF(ab')₂ to Jurkat

cells. Nor did recruitment of these murine residues into anti-CD3 v1 improve Jurkat binding:

V_H K75S (v6), V_H N76S (v7), V_H K75S:N76S (v8), V_L E55H (v11) (not shown). BsF(ab')₂ v9

was chosen for future study since it is amongst the most efficient variants in binding to Jurkat

cells and c ntains fewest murine residues in the humanized anti-CD3 arm. A mon specific anti-p185^{HER2} F(ab')$_2$ did not sh w significant binding to Jurkat cells consistent with the interaction being mediated through the anti-CD3 arm.

DISCUSSION

A minimalistic strategy was chosen to humanize the anti-p185^{HER2} (Carter *et al.*, 1992a, *supra*) and anti-CD3 arms (Shalaby *et al.*, *supra*) of the BsF(ab')$_2$ in this study in an attempt to minimize the potential immunogenicity of the resulting humanized antibody in the clinic. Thus we tried to install the minimum number of murine CDR and FR residues into the context of consensus human variable domain sequences as required to recruit antigen-binding affinity and biological properties comparable to the murine parent antibody. Molecular modeling was used firstly to predict the murine FR residues which might be important to antigen binding and secondly to predict the murine CDR residues that might *not* be required. A small number of humanized variants were then constructed to test these predictions.

Our humanization strategy was very successful for the anti-p185^{HER2} antibody where one out of eight humanized variants (HuMAb4D5-8, IgG1) was identified that bound the p185^{HER2} antigen ~3-fold more tightly than the parent murine antibody (Carter *et al.*, 1992a, *supra*). HuMAb4D5-8 contains a total of five murine FR residues and nine murine CDR residues, including V$_H$ CDR2 residues 60-65, were discarded in favor of human counterparts. In contrast, BsF(ab')$_2$ v1 containing the most potent humanized anti-CD3 variant out of four originally constructed (Shalaby *et al.*, *supra*) binds J6 cells with an affinity (K_d) of 140 nM which is ~70-fold weaker than that of the corresponding chimeric BsF(ab')$_2$.

Here we have restored T cell binding of the humanized anti-CD3 close to that of the chimeric variant by replacing six human residues in V$_H$ CDR2 with their murine counterparts: T57S:A60N:D61Q:S62K:V63F:G65D (anti-CD3 v9, Fig. 5). It appears more likely that these murine residues enhance antigen binding *indirectly* by influencing the conformation of residues in the N-terminal part of V$_H$ CDR2 rather than by *directly* contacting antigen. Firstly, only N-terminal residues in V$_H$ CDR2 (50-58) have been found to contact antigen in one or more of eight crystallographic structures of antibody/antigen complexes (Kabat *et al.*, *supra*; and Mian, I. S. *et al.*, *J. Mol. Biol.* 217: 133-151 (1991), Fig. 5). Secondly, molecular modeling suggests that residues in the C-terminal part of V$_H$ CDR2 are at least partially buried (Fig. 5). BsF(ab')$_2$ v9 binds to SK-BR-3 breast tumor cells with equal efficiency to BsF(ab')$_2$ v1 and chimeric BsF(ab')$_2$ as anticipated since the anti-p185^{HER2} arm is identical in all of these molecules (Shalaby *et al.*, *supra*, not shown).

Our novel approach to the construction of BsF(ab')$_2$ fragments exploits an *E. coli* expression system which secretes humanized Fab' fragments at gram per liter titers and permits their direct recovery as Fab'-SH (Carter *et al.*, 1992b, *supra*). Traditional directed chemical coupling of Fab'-SH fragments is then used to form BsF(ab')$_2$ *in vitro* (Brennan *et*

al.,supra; and Glennie *et al., supra*). This route t Fab'-SH obviates probl ms which are inher nt in their generation from intact antibodies: differences in susceptibility t prote lysis and nonspecific cleavage resulting in heterogeneity, l w yield as well as partial reduction that is n t completely selective for the hinge disulfide bonds. The strategy of using *E. coli*-derived

5   Fab'-SH containing a single hinge cysteine abolishes some sources of heterogeneity in BsF(ab')$_2$ preparation such as intra-hinge disulfide formation and contamination with intact parent antibody whilst greatly diminishes others, eg. formation of F(ab')$_3$ fragments.

BsF(ab')2 fragments constructed here were thioether-linked as originally described by Glennie *et al., supra* with future *in vivo* testing of these molecules in mind. Thioether bonds,

10 unlike disulfide bonds, are not susceptible to cleavage by trace amounts of thiol, which led to the proposal that thioether-linked F(ab')$_2$ may be more stable than disulfide-linked F(ab')$_3$ *in vivo* (Glennie *et al., supra*). This hypothesis is supported by our preliminary pharmacokinetic experiments in normal mice which suggest that thioether-linked BsF(ab')$_3$ v1 has a 3- fold longer plasma residence time than BsF(ab')$_2$ v1 linked by a single disulfide bond.

15 Disulfide and thioether-linked chimeric BsF(ab')$_2$ were found to be indistinguishable in their efficiency of cell binding and in their retargeting of CTL cytotoxicity, which suggests that o-PDM directed coupling does not compromise binding of the BsF(ab')$_2$ to either antigen (not shown). Nevertheless the nature of the linkage appears not to be critical since a disulfide-linked BsF(ab')$_2$ (murine anti-p185^{HER2} / murine anti-CD3) was recently shown by others

20 (Nishimura *et al., Int. J. Cancer* 50: 800-804 (1992) to have potent anti-tumor activity in nude mice. Our previous study (Shalaby *et al., supra*) together with this one and that of Nishimura, T. *et al., supra* improve the potential for using BsF(ab')$_2$ in targeted immunotherapy of_ p185^{HER2}-overexpressing cancers in humans.

25 <u>EXAMPLE 4. Humanization of an anti-CD18 antibody</u>

A murine antibody directed against the leukocyte adhesion receptor β-chain (known as the H52 antibody) was humanized and several versions were created following the methods described above. Figures 6A and 6B provide amino acid sequence comparisons for light chains and heavy chains of the murine and a preferred humanized antibody. Figure 7A shows

30 an amino acid sequence alignment of the sequences of thirteen humanized H52 variants (SEQ. ID. NOs 26-38); Figure 7B shows an amino acid sequence alignment of two humanized H52 light chain variants (SEQ. ID. NOs 39-40). A specific light chain amino acid sequence may be paired with a selected heavy chain amino acid sequence to obtain antibody or antibody fragment molecules with desired binding characteristics.

35

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5   (i) APPLICANT: Genentech, Inc.

(ii) TITLE OF INVENTION: Immunoglobulin Variants

(iii) NUMBER OF SEQUENCES: 40

10
(iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: Genentech, Inc.
        (B) STREET: 460 Point San Bruno Blvd
        (C) CITY: South San Francisco
15   (D) STATE: California
        (E) COUNTRY: USA
        (F) ZIP: 94080

(v) COMPUTER READABLE FORM:
20   (A) MEDIUM TYPE: 5.25 inch, 360 Kb floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: patin (Genentech)

25   (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

30   (vii) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: 07/715272
        (B) FILING DATE: 14-JUN-1991

(vii) PRIOR APPLICATION DATA:
35   (A) APPLICATION NUMBER: PCT/US92/05126
        (B) FILING DATE: 15-JUN-1992

(vii) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: 07/934373
40   (B) FILING DATE: 21-AUG-1992

(viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME:
        (B) REGISTRATION NUMBER:
45   (C) REFERENCE/DOCKET NUMBER: 709P2PCT

(ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE:
        (B) TELEFAX: 415/952-9881
50   (C) TELEX: 910/371-7168

55

(2) INFORMATION FOR SEQ ID NO:1:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 109 amin   acids
      (B) TYPE: amin   acid
      (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1   5   10   15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn
         20   25   30

Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
         35   40   45

Leu Leu Ile Tyr Ser Ala Ser Phe Leu Glu Ser Gly Val Pro Ser
         50   55   60

Arg Phe Ser Gly Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile
         65   70   75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
         80   85   90

His Tyr Thr Thr Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
         95   100   105

Ile Lys Arg Thr
         109

(2) INFORMATION FOR SEQ ID NO:2:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 120 amino acids
      (B) TYPE: amino acid
      (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1   5   10   15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys
         20   25   30

Asp Thr Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
         35   40   45

Glu Trp Val Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
         50   55   60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser
         65   70   75

Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
       80   85   90

Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
       95   100   105

Ala Met Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
      110   115   120

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 109 amino acids
    (B) TYPE: amino acid
    (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1   5   10   15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Ser
       20   25   30

Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
       35   40   45

Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
       50   55   60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
       65   70   75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
       80   85   90

Tyr Asn Ser Leu Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu
       95   100   105

Ile Lys Arg Thr
      109

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 120 amino acids
    (B) TYPE: amino acid
    (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1   5   10   15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
       20   25   30

Asp Tyr Ala Met Ser Trp Val Arg Gln Ala Pr Gly Lys Gly Leu
         35   40   45

Glu Trp Val Ala Val Ile Ser Glu Asn Gly Gly Tyr Thr Arg Tyr
         50   55   60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser
         65   70   75

Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
         80   85   90

Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
         95   100   105

Ala Met Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
         110   115   120

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 109 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Asp Ile Val Met Thr Gln Ser His Lys Phe Met Ser Thr Ser Val
 1   5   10   15

Gly Asp Arg Val Ser Ile Thr Cys Lys Ala Ser Gln Asp Val Asn
         20   25   30

Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly His Ser Pro Lys
         35   40   45

Leu Leu Ile Tyr Ser Ala Ser Phe Arg Tyr Thr Gly Val Pro Asp
         50   55   60

Arg Phe Thr Gly Asn Arg Ser Gly Thr Asp Phe Thr Phe Thr Ile
         65   70   75

Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln
         80   85   90

His Tyr Thr Thr Pro Pro Thr Phe Gly Gly Gly Thr Lys Leu Glu
         95   100   105

Ile Lys Arg Ala
         109

(2) INFORMATION FOR SEQ ID NO:6:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 120 amino acids
      (B) TYPE: amino acid
      (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
1   5   10   15

Ala Ser Leu Lys Leu Ser Cys Thr Ala Ser Gly Phe Asn Ile Lys
       20   25   30

Asp Thr Tyr Ile His Trp Val Lys Gln Arg Pro Glu Gln Gly Leu
       35   40   45

Glu Trp Ile Gly Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
       50   55   60

Asp Pro Lys Phe Gln Asp Lys Ala Thr Ile Thr Ala Asp Thr Ser
       65   70   75

Ser Asn Thr Ala Tyr Leu Gln Val Ser Arg Leu Thr Ser Glu Asp
       80   85   90

Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
       95   100   105

Ala Met Asp Tyr Trp Gly Gln Gly Ala Ser Val Thr Val Ser Ser
       110   115   120

(2) INFORMATION FOR SEQ ID NO:7:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 27 bases
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

     TCCGATATCC AGCTGACCCA GTCTCCA 27

(2) INFORMATION FOR SEQ ID NO:8:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 31 bases
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

     GTTTGATCTC CAGCTTGGTA CCHSCDCCGA A 31

(2) INFORMATION FOR SEQ ID NO:9:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 22 bases
5   (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
10

     AGGTSMARCT GCAGSAGTCW GG 22

(2) INFORMATION FOR SEQ ID NO:10:

15   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 34 bases
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear
20

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

     TGAGGAGACG GTGACCGTGG TCCCTTGGCC CCAG 34

25 (2) INFORMATION FOR SEQ ID NO:11:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 36 bases
     (B) TYPE: nucleic acid
30   (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

35   GTAGATAAAT CCTCTAACAC AGCCTATCTG CAAATG 36

(2) INFORMATION FOR SEQ ID NO:12:

   (I) SEQUENCE CHARACTERISTICS:
40   (A) LENGTH: 36 bases
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

45   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

     GTAGATAAAT CCAAATCTAC AGCCTATCTG CAAATG 36

(2) INFORMATION FOR SEQ ID NO:13:
50
   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 36 bases
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
55   (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTAGATAAAT CCTCTTCTAC AGCCTATCTG CAAATG 36

5   (2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 bases
(B) TYPE: nucleic acid
10   (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

15   CTTATAAAGG TGTTTCCACC TATAACCAGA AATTCAAGGA TCGTTTCACG 50

ATATCCGTAG ATAAATCC 68

(2) INFORMATION FOR SEQ ID NO:15:
20

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
25   (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CTATACCTCC CGTCTGCATT CTGGAGTCCC 30
30
(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
35   (B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

40   Asp Ile Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu
        1   5   10   15

Gly Asp Arg Val Thr Ile Ser Cys Arg Ala Ser Gln Asp Ile Arg
                20   25   30
45
Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Asp Gly Thr Val Lys
                35   40   45

Leu Leu Ile Tyr Tyr Thr Ser Arg Leu His Ser Gly Val Pro Ser
50   50   55   60

Lys Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile
                65   70   75

55   Ser Asn Leu Glu Gln Glu Asp Ile Ala Thr Tyr Phe Cys Gln Gln
                80   85   90

Gly Asn Thr Leu Pro Trp Thr Phe Ala Gly Gly Thr Lys Leu Glu
       95   100   105

Ile Lys
  107

(2) INFORMATION FOR SEQ ID NO:17:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 107 amino acids
    (B) TYPE: amino acid
    (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
  1   5   10   15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Ile Arg
      20   25   30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
      35   40   45

Leu Leu Ile Tyr Tyr Thr Ser Arg Leu Glu Ser Gly Val Pro Ser
      50   55   60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile
      65   70   75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
      80   85   90

Gly Asn Thr Leu Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
      95   100   105

Ile Lys
  107

(2) INFORMATION FOR SEQ ID NO:18:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 107 amino acids
    (B) TYPE: amino acid
    (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
  1   5   10   15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser
      20   25   30

Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
      35   40   45

L u Leu ll Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
           50   55   60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
5   65   70   75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
           80   85   90

10 Tyr Asn Ser Leu Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
           95   100   105

Ile Lys
   107
15

(2) INFORMATION FOR SEQ ID NO:19:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 129 amino acids
20   (B) TYPE: amino acid
      (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

25 Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
     1   5   10   15

Ala Ser Met Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ser Phe Thr
         20   25   30
30

Gly Tyr Thr Met Asn Trp Val Lys Gln Ser His Gly Lys Asn Leu
         35   40   45

Glu Trp Met Gly Leu Ile Asn Pro Tyr Lys Gly Val Ser Thr Tyr
35   50   55   60

Asn Gln Lys Phe Lys Asp Arg Phe Thr Ile Ser Lys Ala Thr Leu
         65   70   75

40 Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr Leu Met Glu Leu Leu
         80   85   90

Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys Ala Arg
         95   100   105
45

Ser Gly Tyr Tyr Gly Asp Ser Asp Trp Tyr Phe Asp Val Trp Gly
        110   115   120

Ala Gly Thr Thr Val Thr Val Ser Ser
50   125   129

55

(2) INFORMATION FOR SEQ ID NO:20:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 122 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1           5              10           15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr
          20           25           30

Gly Tyr Thr Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
          35           40           45

Glu Trp Val Ala Leu Ile Asn Pro Tyr Lys Gly Val Ser Thr Tyr
          50           55           60

Asn Gln Lys Phe Lys Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
          65           70           75

Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
          80           85           90

Thr Ala Val Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser
          95           100          105

Asp Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val
          110          115          120

Ser Ser
  122
```

(2) INFORMATION FOR SEQ ID NO:21:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 122 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1           5              10           15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
          20           25           30

Ser Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
          35           40           45

Glu Trp Val Ser Val Ile Ser Gly Asp Gly Gly Ser Thr Tyr Tyr
          50           55           60
```

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
      65   70   75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
      80   85   90

Thr Ala Val Tyr Tyr Cys Ala Arg Gly Arg Val Gly Tyr Ser Leu
      95   100   105

Ser Gly Leu Tyr Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val
      110   115   120

Ser Ser
122

(2) INFORMATION FOR SEQ ID NO:22:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 454 amino acids
    (B) TYPE: amino acid
    (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
1   5   10   15

Ala Ser Val Lys Ile Ser Cys Lys Thr Ser Gly Tyr Thr Phe Thr
      20   25   30

Glu Tyr Thr Met His Trp Met Lys Gln Ser His Gly Lys Ser Leu
      35   40   45

Glu Trp Ile Gly Gly Phe Asn Pro Lys Asn Gly Gly Ser Ser His
      50   55   60

Asn Gln Arg Phe Met Asp Lys Ala Thr Leu Ala Val Asp Lys Ser
      65   70   75

Thr Ser Thr Ala Tyr Met Glu Leu Arg Ser Leu Thr Ser Glu Asp
      80   85   90

Ser Gly Ile Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
      95   100   105

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Ala Gly Thr Thr Val
      110   115   120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
      125   130   135

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
      140   145   150

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
      155   160   165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
            170   175   180

5   Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
            185   190   195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
            200   205   210

10   His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
            215   220   225

Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu
            230   235   240

15
Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
            245   250   255

Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
20   260   265   270

Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr
            275   280   285

25   Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
            290   295   300

Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val
            305   310   315

30
Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val
            320   325   330

Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys
35   335   340   345

Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
            350   355   360

40   Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
            365   370   375

Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
            380   385   390

45
Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu
            395   400   405

Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
50   410   415   420

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
            425   430   435

55   His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
            440   445   450

-86-

Ser Pro Gly Lys
            454

(2) INFORMATION FOR SEQ ID NO:23:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 557 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

His His Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
 1   5   10   15

Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Thr Ser Gly Tyr Thr
            20   25   30

Phe Thr Glu Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala
            35   40   45

Thr Ala Thr Gly Val His Ser Glu Val Gln Leu Val Glu Ser Gly
            50   55   60

Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala
            65   70   75

Thr Ser Gly Tyr Thr Phe Thr Glu Tyr Thr Met His Trp Met Arg
            80   85   90

Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala Gly Ile Asn Pro
            95   100   105

Lys Asn Gly Gly Thr Ser His Asn Gln Arg Phe Met Asp Arg Phe
            110   115   120

Thr Ile Ser Val Asp Lys Ser Thr Ser Thr Ala Tyr Met Gln Met
            125   130   135

Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg
            140   145   150

Trp Arg Gly Leu Asn Tyr Gly Phe Asp Val Arg Tyr Phe Asp Val
            155   160   165

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys
            170   175   180

Gly Pro Ser Val Phe Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser
            185   190   195

Glu Ser Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro
            200   205   210

Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
            215   220   225

-87-

Val His Thr Phe Pro Ala Val Leu Gln Ser S r Gly Leu Tyr Ser
                230   235   240

Leu Ser Ser Val Val Thr Val Thr Ser Ser Asn Phe Gly Thr Gln
    5   245   250   255

Thr Tyr Thr Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val
                260   265   270

   10   Asp Lys Thr Val Glu Arg Lys Cys Cys Val Thr Cys Pro Pro Cys
                275   280   285

Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro
                290   295   300
   15

Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val
                305   310   315

Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys
   20   320   325   330

Glu Cys Pro Pro Cys Pro Ala Pro Pro Val Ala Gly Pro Ser Val
                335   340   345

   25   Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg
                350   355   360

Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp
                365   370   375
   30

Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Met Glu Val His
                380   385   390

Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe
   35   395   400   405

Arg Val Val Ser Val Leu Thr Val Val His Gln Asp Trp Leu Asn
                410   415   420

   40   Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro Ala
                425   430   435

Pro Ile Glu Lys Thr Ile Ser Lys Thr Lys Gly Gln Pro Arg Glu
                440   445   450
   45

Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys
                455   460   465

Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser
   50   470   475   480

Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn
                485   490   495

   55   Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly Ser Phe Phe
                500   505   510

-88-

Leu Tyr Ser Lys Leu Thr Val Asp Lys S r Arg Trp Gln Gln Gly
            515   520   525

Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His
            530   535   540

Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
            545   550   555

(2) INFORMATION FOR SEQ ID NO:24:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 214 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Asp Val Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu
  1   5   10   15

Gly Asp Arg Val Thr Ile Asn Cys Arg Ala Ser Gln Asp Ile Asn
            20   25   30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Asn Gly Thr Val Lys
            35   40   45

Leu Leu Ile Tyr Tyr Thr Ser Thr Leu His Ser Gly Val Pro Ser
            50   55   60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile
            65   70   75

Ser Asn Leu Asp Gln Glu Asp Ile Ala Thr Tyr Phe Cys Gln Gln
            80   85   90

Gly Asn Thr Leu Pro Pro Thr Phe Gly Gly Gly Thr Lys Val Glu
            95   100   105

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
            110   115   120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
            125   130   135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
            140   145   150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
            155   160   165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
            170   175   180

Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
            185   190   195

Val Thr His Gln Gly Leu Ser Ser Pr   Val Thr Lys Ser Phe Asn
                200   205   210

Arg Gly Glu Cys
                214

(2) INFORMATION FOR SEQ ID NO:25:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 233 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr
 1   5   10   15

Gly Val His Ser Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu
            20   25   30

Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser
            35   40   45

Gln Asp Ile Asn Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly
            50   55   60

Lys Ala Pro Lys Leu Leu Ile Tyr Tyr Thr Ser Thr Leu His Ser
            65   70   75

Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr
            80   85   90

Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr
            95   100   105

Tyr Cys Gln Gln Gly Asn Thr Leu Pro Pro Thr Phe Gly Gln Gly
            110   115   120

Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe
            125   130   135

Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser
            140   145   150

Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val
            155   160   165

Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu
            170   175   180

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
            185   190   195

Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val
            200   205   210

Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr
       215   220   225

Lys Ser Phe Asn Arg Gly Glu Cys
       230   233

(2) INFORMATION FOR SEQ ID NO:26:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 232 amino acids
      (B) TYPE: amino acid
      (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1   5   10   15

Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Tyr Thr Phe Thr
      20   25   30

Glu Tyr Thr Met His Trp Met Arg Gln Ala Pro Gly Lys Gly Leu
      35   40   45

Glu Trp Val Ala Gly Ile Asn Pro Lys Asn Gly Gly Thr Ser Tyr
      50   55   60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser
      65   70   75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
      80   85   90

Thr Ala Val Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
      95   100   105

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val
      110   115   120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
      125   130   135

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
      140   145   150

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
      155   160   165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
      170   175   180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
      185   190   195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
      200   205   210

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pr Lys
            215   220   225 .

Ser Cys Asp Lys Thr His Thr
            230 ` 232

(2) INFORMATION FOR SEQ ID NO:27:

   (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 232 amino acids
       (B) TYPE: amino acid
       (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1   5   10   15

Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Tyr Thr Phe Thr
            20   25   30

Glu Tyr Thr Met His Trp Met Arg Gln Ala Pro Gly Lys Gly Leu
            35   40   45

Glu Trp Val Ala Gly Ile Asn Pro Lys Asn Gly Gly Thr Ser His
            50   55   60

Asn Gln Arg Phe Met Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
            65   70   75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
            80   85   90

Thr Ala Val Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
            95   100   105

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val
            110   115   120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
            125   130   135

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
            140   145   150

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
            155   160   165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
            170   175   180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
            185   190   195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
            200   205   210

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
215   220   225

S r Cys Asp Lys Thr His Thr
230   232

(2) INFORMATION FOR SEQ ID NO:28:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 232 amino acids
    (B) TYPE: amino acid
    (D) TOPOLOGY: linear

 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1   5   10   15

Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Tyr Thr Phe Thr
20   25   30

Glu Tyr Thr Met His Trp Met Arg Gln Ala Pro Gly Lys Gly Leu
35   40   45

Glu Trp Val Ala Gly Ile Asn Pro Lys Asn Gly Gly Thr Ser His
50   55   60

Asn Gln Arg Phe Met Asp Arg Phe Thr Leu Ala Val Asp Lys Ser
65   70   75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
80   85   90

Thr Ala Val Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
95   100   105

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val
110   115   120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
125   130   135

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
140   145   150

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
155   160   165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
170   175   180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
185   190   195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
200   205   210

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
        215   220   225

Ser Cys Asp Lys Thr His Thr
        230   232

(2) INFORMATION FOR SEQ ID NO:29:

  (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 232 amino acids
     (B) TYPE: amino acid
     (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1   5   10   15

Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Tyr Thr Phe Thr
        20   25   30

Glu Tyr Thr Met His Trp Met Arg Gln Ala Pro Gly Lys Gly Leu
        35   40   45

Glu Trp Val Ala Gly Ile Asn Pro Lys Asn Gly Gly Thr Ser His
        50   55   60

Asn Gln Arg Phe Met Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
        65   70   75

Thr Ser Thr Ala Tyr Met Gln Met Asn Ser Leu Arg Ala Glu Asp
        80   85   90

Thr Ala Val Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
        95   100   105

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val
        110   115   120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
        125   130   135

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
        140   145   150

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
        155   160   165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
        170   175   180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
        185   190   195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        200   205   210

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
            215   220   225

Ser Cys Asp Lys Thr His Thr
            230   232

(2) INFORMATION FOR SEQ ID NO:30:

   (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 235 amino acids
       (B) TYPE: amino acid
       (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
    1   5   10   15

Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Tyr Thr Phe Thr
            20   25   30

Glu Tyr Thr Met His Trp Met Arg Gln Ala Pro Gly Lys Gly Leu
            35   40   45

Glu Trp Val Ala Gly Ile Asn Pro Lys Asn Gly Gly Thr Ser His
            50   55   60

Asn Gln Arg Phe Met Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
            65   70   75

Thr Ser Thr Ala Tyr Met Gln Met Asn Ser Leu Arg Ala Glu Asp
            80   85   90

Thr Ala Val Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
            95   100   105

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val
            110   115   120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
            125   130   135

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
            140   145   150

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
            155   160   165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
            170   175   180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
            185   190   195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
            200   205   210

His Lys Pr Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pr Lys
            215   220   225

Ser Cys Asp Lys Thr His Thr Cys Pr Pro
            230   235

(2) INFORMATION FOR SEQ ID NO:31:

  (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 232 amino acids
      (B) TYPE: amino acid
      (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1   5   10   15

Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Tyr Thr Phe Thr
            20   25   30

Glu Tyr Thr Met His Trp Met Arg Gln Ala Pro Gly Lys Gly Leu
            35   40   45

Glu Trp Val Ala Gly Ile Asn Pro Lys Asn Gly Gly Thr Ser His
            50   55   60

Asn Gln Arg Phe Met Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
            65   70   75

Lys Asn Thr Leu Tyr Met Glu Leu Arg Ser Leu Arg Ala Glu Asp
            80   85   90

Thr Ala Val Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
            95   100   105

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val
            110   115   120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
            125   130   135

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
            140   145   150

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
            155   160   165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
            170   175   180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
            185   190   195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
            200   205   210

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
                215   220   225

Ser Cys Asp Lys Thr His Thr
                230   232

(2) INFORMATION FOR SEQ ID NO:32:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 232 amino acids
      (B) TYPE: amino acid
      (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
   1   5   10   15

Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Tyr Thr Phe Thr
                20   25   30

Glu Tyr Thr Met His Trp Met Arg Gln Ala Pro Gly Lys Gly Leu
                35   40   45

Glu Trp Val Ala Gly Ile Asn Pro Lys Asn Gly Gly Thr Ser His
                50   55   60

Asn Gln Arg Phe Met Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
                65   70   75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Thr Ser Glu Asp
                80   85   90

Ser Gly Ile Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
                95   100   105

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val
                110   115   120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
                125   130   135

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
                140   145   150

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
                155   160   165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
                170   175   180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
                185   190   195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
                200   205   210

His Lys Pr Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pr Lys
            215   220   225

Ser Cys Asp Lys Thr His Thr
            230   232

(2) INFORMATION FOR SEQ ID NO:33:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 232 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1   5   10   15

Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Tyr Thr Phe Thr
            20   25   30

Glu Tyr Thr Met His Trp Met Arg Gln Ala Pro Gly Lys Gly Leu
            35   40   45

Glu Trp Val Ala Gly Ile Asn Pro Lys Asn Gly Gly Thr Ser His
            50   55   60

Asn Gln Arg Phe Met Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
            65   70   75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
            80   85   90

Thr Ala Val Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
            95   100   105

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Ala Gly Thr Thr Val
            110   115   120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
            125   130   135

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
            140   145   150

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
            155   160   165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
            170   175   180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
            185   190   195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
            200   205   210

His Lys Pr S r Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
            215   220   225

Ser Cys Asp Lys Thr His Thr
            230   232

(2) INFORMATION FOR SEQ ID NO:34:

   (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 232 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Gln Pro Gly
    1   5   10   15

Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Tyr Thr Phe Thr
            20   25   30

Glu Tyr Thr Met His Trp Met Arg Gln Ala Pro Gly Lys Gly Leu
            35   40   45

Glu Trp Val Ala Gly Ile Asn Pro Lys Asn Gly Gly Thr Ser His
            50   55   60

Asn Gln Arg Phe Met Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
            65   70   75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
            80   85   90

Thr Ala Val Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
            95   100   105

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val
            110   115   120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
            125   130   135

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
            140   145   150

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
            155   160   165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
            170   175   180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
            185   190   195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
            200   205   210

-99-

His Lys Pr Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
        215   220   225

Ser Cys Asp Lys Thr His Thr
        230   232

(2) INFORMATION FOR SEQ ID NO:35:

  (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 232 amino acids
     (B) TYPE: amino acid
     (D) TOPOLOGY: linear

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Lys Pro Gly
  1   5   10   15

Ala Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Tyr Thr Phe Thr
        20   25   30

Glu Tyr Thr Met His Trp Met Arg Gln Ala Pro Gly Lys Gly Leu
        35   40   45

Glu Trp Val Ala Gly Ile Asn Pro Lys Asn Gly Gly Thr Ser His
        50   55   60

Asn Gln Arg Phe Met Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
        65   70   75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
        80   85   90

Thr Ala Val Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
        95   100   105

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val
        110   115   120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
        125   130   135

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
        140   145   150

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
        155   160   165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
        170   175   180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
        185   190   195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        200   205   210

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
215   220   225

Ser Cys Asp Lys Thr His Thr
230   232

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 232 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1   5   10   15

Gly Ser Leu Lys Ile Ser Cys Lys Thr Ser Gly Tyr Thr Phe Thr
20   25   30

Glu Tyr Thr Met His Trp Met Arg Gln Ala Pro Gly Lys Gly Leu
35   40   45

Glu Trp Val Ala Gly Ile Asn Pro Lys Asn Gly Gly Thr Ser His
50   55   60

Asn Gln Arg Phe Met Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
65   70   75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
80   85   90

Thr Ala Val Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
95   100   105

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val
110   115   120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
125   130   135

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
140   145   150

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
155   160   165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
170   175   180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
185   190   195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
200   205   210

His Lys Pr Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
            215   220   225

Ser Cys Asp Lys Thr His Thr
            230   232

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 232 amino acids
    (B) TYPE: amino acid
    (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1   5   10   15

Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Tyr Thr Phe Thr
            20   25   30

Glu Tyr Thr Met His Trp Met Lys Gln Ser His Gly Lys Ser Leu
            35   40   45

Glu Trp Val Ala Gly Ile Asn Pro Lys Asn Gly Gly Thr Ser His
            50   55   60

Asn Gln Arg Phe Met Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
            65   70   75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
            80   85   90

Thr Ala Val Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
            95   100   105

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val
            110   115   120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
            125   130   135

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
            140   145   150

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
            155   160   165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
            170   175   180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
            185   190   195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
            200   205   210

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
          215   220   225

Ser Cys Asp Lys Thr His Thr
          230   232

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 232 amino acids
    (B) TYPE: amino acid
    (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1   5   10   15

Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Tyr Thr Phe Thr
          20   25   30

Glu Tyr Thr Met His Trp Met Arg Gln Ala Pro Gly Lys Gly Leu
          35   40   45

Glu Trp Ile Gly Gly Phe Asn Pro Lys Asn Gly Gly Thr Ser His
          50   55   60

Asn Gln Arg Phe Met Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
          65   70   75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
          80   85   90

Thr Ala Val Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
          95   100   105

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val
          110   115   120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
          125   130   135

Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
          140   145   150

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
          155   160   165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
          170   175   180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
          185   190   195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
          200   205   210

-103-

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pr Lys
          215   220   225   .

Ser Cys Asp Lys Thr His Thr
5   230 · 232

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
10   (A) LENGTH: 214 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:
15
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 .1   5   10   15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Ile Asn
20   20   25   30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
          35   40   45

25   Leu Leu Ile Tyr Tyr Thr Ser Thr Leu Glu Ser Gly Val Pro Ser
          50   55   60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile
          65   70   75
30
Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
          80   85   90

Gly Asn Thr Leu Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
35   95   100   105   ·

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
          110   115   120

40   Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
          125   130   135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
          140   145   150
45
Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
          155   160   165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
50   170   175   180

Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
          185   190   195

55   Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
        · 200   205   210

Arg Gly Glu Cys
        214

(2) INFORMATION FOR SEQ ID NO:40:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 214 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1   5   10   15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Ile Asn
             20   25   30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
             35   40   45

Leu Leu Ile Tyr Tyr Thr Ser Thr Leu His Ser Gly Val Pro Ser
             50   55   60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile
             65   70   75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
             80   85   90

Gly Asn Thr Leu Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
             95   100   105

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
             110   115   120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
             125   130   135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
             140   145   150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
             155   160   165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
             170   175   180

Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
             185   190   195

Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
             200   205   210

Arg Gly Glu Cys
        214

CLAIMS

WE CLAIM:

1.   A method for making a humanized antibody comprising amino acid sequence of a non-
     human, import antibody and a human antibody, comprising the steps of:

     a.   obtaining the amino acid sequences of at least a portion of an import variable
          domain and of a consensus human variable domain;

     b.   Identifying Complementarity Determining Region (CDR) amino acid sequences in
          the import and the human amino variable domain sequences;

     c.   substituting an import CDR amino acid sequence for the corresponding human
          CDR amino acid sequence;

     d.   aligning the amino acid sequences of a Framework Region (FR) of the import
          antibody and the corresponding FR of the consensus antibody;

     e.   identifying import antibody FR residues in the aligned FR sequences that are non-
          homologous to the corresponding consensus antibody residues;

     f.   determining if the non-homologous import amino acid residue is reasonably
          expected to have at least one of the following effects:

          1.   non-covalently binds antigen directly,

          2.   interacts with a CDR; or

          3.   participates in the V_L - V_H interface; and

     g.   for any non-homologous import antibody amino acid residue which is reasonably
          expected to have at least one of these effects, substituting that residue for the
          corresponding amino acid residue in the consensus antibody FR sequence.

2.   The method of claim 1, having an additional step of determining if any such non-
     homologous residues are exposed on the surface of the domain or buried within it, and
     if the residue is exposed, retaining the consensus residue.

3.   The method of claim 1, having the additional steps of searching the import variable
     domain sequence for glycosylation sites, determining if any such glycosylation site is
     reasonably expected to affect the antigen binding or affinity of the antibody, and if so,
     substituting the glycosylation site into the consensus sequence.

4.   The method of claim 1, having the additional steps of searching the consensus variable
     domain sequence for glycosylation sites which are not present at the corresponding
     amino acid in the import sequence, and if the glycosylation site is not present in the
     import sequence, substituting the import amino acid residues for the amino acid
     residues comprising the consensus glycosylation site.

5.   The method of claim 1, having an additional step which comprises aligning import
     antibody and consensus antibody FR sequences, identifying import antibody FR
     residues which are non-homologous with the aligned consensus FR sequence, and for
     each such non-homologous import antibody FR residue, determining if the

-106-

corresponding consensus antibody residue represents a residue which is highly
c nserved across all sp cies at that site, and if it is so conserved, preparing a
humanized antibody which c mprises the c nsensus antibody amino acid residue at
that site.

5 6.   The method of claim 1, wherein the corresponding consensus antibody residues are
       selected from the group consisting of 4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L,
       63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71L, 73L, 85L, 87L, 98L, 2H, 4H, 24H, 36H,
       37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H,
       78H, 91H, 92H, 93H, and 103H.

10 7. A method comprising providing at least a portion of an import, non-human antibody
       variable domain amino acid sequence having a CDR and a FR, obtaining the amino acid
       sequence of at least a portion of a consensus human antibody variable domain having
       a CDR and a FR, substituting the non-human CDR for the human CDR in the consensus
       human antibody variable domain, and then substituting an amino acid residue for the
15   consensus amino acid residue at at least one of the following sites:
       4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L,
       71L, 73L, 85L, 87L, 98L, 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H,
       67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H.

   8. The method of claim 7, wherein the substituted residue is the residue found at the
20   corresponding location of the non-human antibody.

   9. The method of claim 1 or 7, wherein the consensus human variable domain is a
       consensus based on human variable domains and additionally variable domains from
       species other than human.

   10. A humanized antibody variable domain having a non-human CDR incorporated into a
25   human antibody variable domain, wherein the improvement comprises substituting an
       amino acid residue for the human residue at a site selected from the group consisting
       of:
       4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L,
       71L, 73L, 85L, 87L, 98L, 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H,
30   67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H.

   11. The humanized antibody variable domain of claim 10, wherein the substituted residue
       is the residue found at the corresponding location of the non-human antibody from
       which the non-human CDR was obtained.

   12. The humanized antibody variable domain of claim 10, wherein no human FR residue
35   other than those set forth in the group has been substituted.

   13. A polypeptide comprising the amino acid sequence:
       DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLESGVP
       SRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRT

-107-

14. A p lypeptide comprising the sequence:
    EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTR
    YADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDVWGQGTLV
    TVSS

15. A method for engineering a humanized antibody comprising introducing amino acid
    residues from an import antibody variable domain into an amino acid sequence
    representing a consensus of mammalian antibody variable domain sequences.

16. A computer comprising the sequence data of the following amino acid sequence:

    a.   DIQMTQSPSSLSASVGDRVTITCRASQDVSSYLAWYQQKPGKAPKLLIYAASSLE
         SGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSLPYTFGQGTKVEIKRT, or

    b.   EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSWVRQAPGKGLEWVAVISE
         NGGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYA
         MDVWGQGTLVTVSS

17. A computer representation of the following amino acid sequence:

    a.   DIQMTQSPSSLSASVGDRVTITCRASQDVSSYLAWYQQKPGKAPKLLIYAASSLE
         SGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSLPYTFGQGTKVEIKRT, or

    b.   EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSWVRQAPGKGLEWVAVISE
         NGGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYA
         MDVWGQGTLVTVSS

18. A method comprising storing a computer representation of the following amino acid
    sequence:

    a.   DIQMTQSPSSLSASVGDRVTITCRASQDVSSYLAWYQQKPGKAPKLLIYAASSLE
         SGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSLPYTFGQGTKVEIKRT, or

    b.   EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSWVRQAPGKGLEWVAVISE
         NGGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYA
         MDVWGQGTLVTVSS

FIG. 1A

FIG. 1B

```
                  10        20        30        40        50 A
4D5       EVQLQQSGPELVKPGASLKLSCTASGFNIKDTYIHWVKQRPEQGLEWIGRIYPTN
HU4D5     EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTN
HUVHIII   EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSWVRQAPGKGLEWVAVISENG
                                   VH-CDR1            VH-CDR2

                  60        70        80 ABC     90       100ABC
4D5       GYTRYDPKFQDKATITADTSSNTAYLQVSRLTSEDTAVYYCSRWGGDGFYAMDYW
HU4D5     GYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDVW
HUVHIII   SDTYYADSVKGRFTISRDDSKNTLYLQMNSLRAEDTAVYYCARDRGGAVSYFDVW
                                                   VH-CDR3

                  110
4D5       GQGASVTVSS
HU4D5     GQGTLVTVSS
HUVHIII   GQGTLVTVSS
```
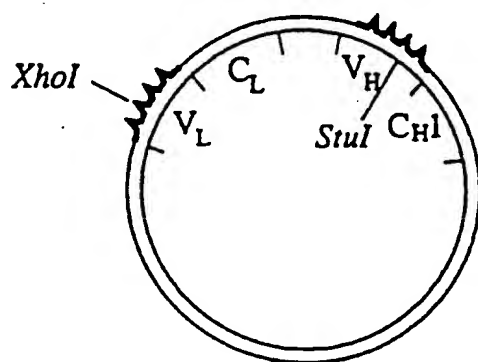
Anneal huV_L or huV_H oligomers to pAK1 template

1. Ligate
2. Isolate assembled oligomers
3. Anneal to pAK1 template ($XhoI^-$, $StuI^+$)
4. Extend and ligate

1. Transform *E. coli*
2. Isolate phagemid pool
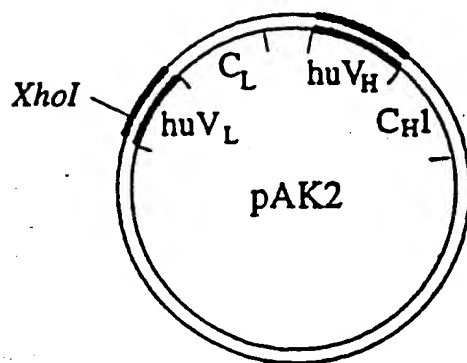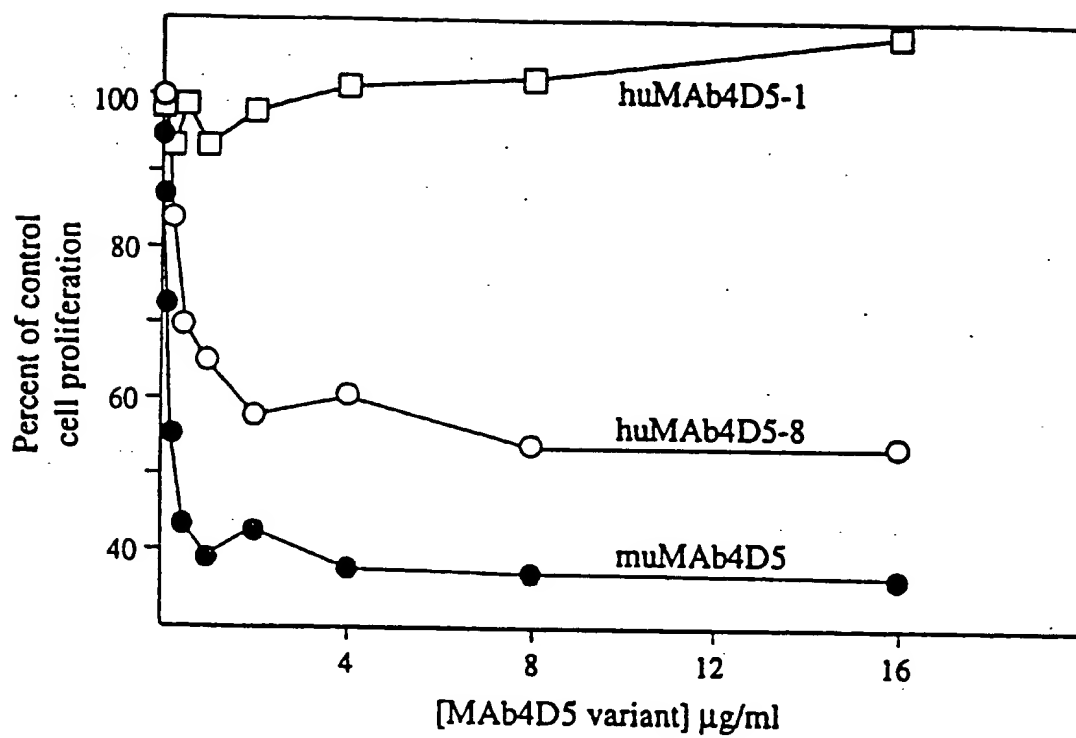3. Enrich for huV_L and huV_H($Xho I^+$, $StuI^-$)
4. Sequence verify

FIG. 2

FIG. 3

FIG. 4

SUBSTITUTE SHEET

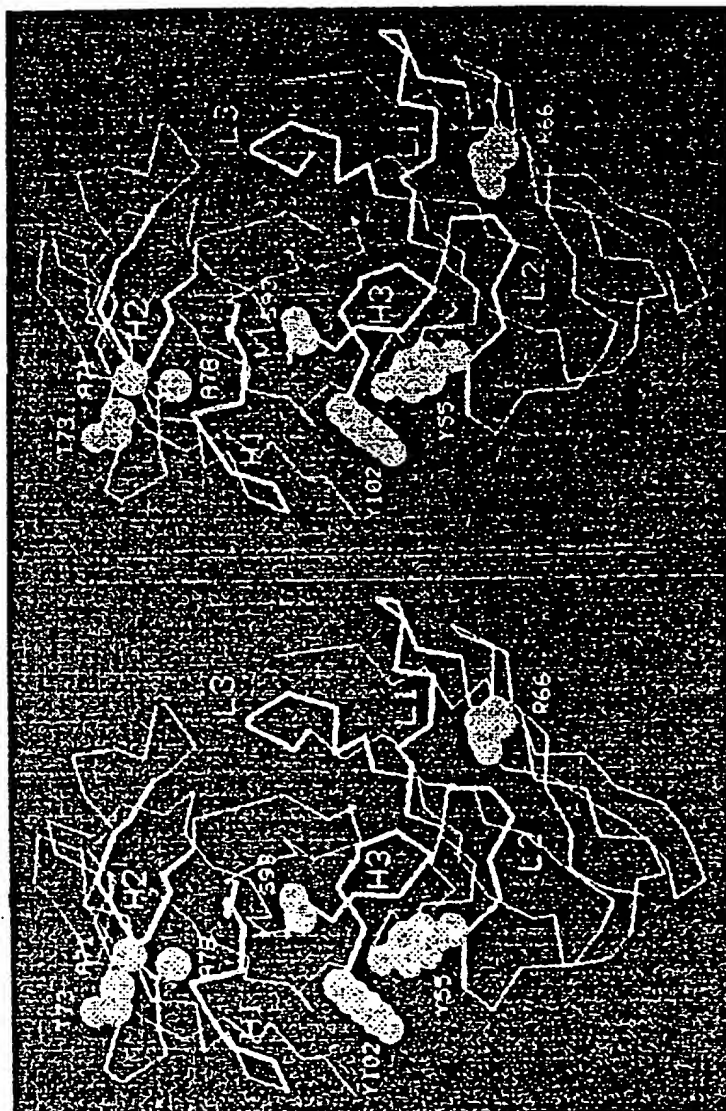V_L

```
              10        20        30        40
muxCD3   DIQMTQTTSSLSASLGDRVTISCRASQDIRNYLNWYQQKP
              **         *         *
huxCD3v1 DIQMTQSPSSLSASVGDRVTITCRASQDIRNYLNWYQQKP
                                     # #        #
huκI     DIQMTQSPSSLSASVGDRVTITCRASQSISNYLAWYQQKP6
                                    ^^^^^^^^
                                    CDR-L1
```

```
              50        60        70        80
muxCD3   DGTVKLLIYYTSRLHSGVPSKFSGSGSGTDYSLTISNLEQ
         ****         *         *           *     *  **
huxCD3v1 GKAPKLLIYYTSRLESGVPSRFSGSGSGTDYTLTISSLQP
                # #                              #
huκI     GKAPKLLIYAASSLESGVPSRFSGSGSGTDFTLTISSLQP
                  ^^^^^
                  CDR-L2
```

```
              90        100
muxCD3   EDIATYFCQQGNTLPWTFAGGTKLEIK
         *       * **         *
huxCD3v1 EDFATYYCQQGNTLPWTFGQGTKVEIK
                  # #
huκI     EDFATYYCQQYNSLPWTFGQGTKVEIK
                 ^^^^^^^^
                 CDR-L3
```

V_H

```
              10        20        30        40
muxCD3   EVQLQQSGPELVKPGASMKISCKASGYSFTGYTMNWVKQS
         **   -**   *    *  ***      *           * *
huxCD3v1 EVQLVESGGGLVQPGGSLRLSCAASGYSFTGYTMNWVRQA
                                     ##  ##  #  #
huIII    EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQA
                                        ^^^^^^^
                                        CDR-H1
```

```
              50        60        70
muxCD3   HGKNLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSSTAY
         *    *      **       *    ** **        **
huxCD3v1 PGKGLEWVALINPYKGVTTYADSVKGRFTISVDKSKNTAY
                   ## ####  # #                    #
HuIII    PGKGLEWVSVISGDGGSTYYADSVKGRFTISRDNSKNTLY
                   ^^^^^
                   CDR-H2
```

```
         80  abc    90    100abcde   110
muxCD3   MELLSLTSEDSAVYYCARSGYYGDSDWYFDVWGAGTTVTVSS
         ****   **   *                       *    *
huxCD3v1 LQMNSLRAEDTAVYYCARSGYYGDSDWYFDVWGQGTLVTVSS
                          ########### #
huIII    LQMNSLRAEDTAVYYCARGRVGYSLSGLYDYWGQGTLVTVSS
                           D ET    S
                          ^^^^^^^^^^^^
                          CDR-H3
```

FIG. 5

FIG. 6A-1

	10 20 30
H52H4-160	QVQLQQSGPELVKPGASVKISCKTSGYTFTE
	.*** .** **.**.*..** *******
pH52-8.0	MGWSCIILFLVATATGVHSEVQLVESGGGLVQPGGSLRLSCATSGYTFTE
	10 20 30 40 50

	40 50 60 70 80
H52H4-160	YTMHWMKQSHGKSLEWIGGFNPKNGGSSHNQRFMDKATLAVDKSTSTAYM
	.*. *. **.*..*.*.**.***. *. .***********.***
pH52-8.0	YTMHWMRQAPGKGLEWVAGINPKNGGTSHNQRFMDRFTISVDKSTSTAYM
	60 70 80 90 100

	90 100 110 120 130
H52H4-160	ELRSLTSEDSGIYYCARWRGLNYGFDVRYFDVWGAGTTVTVSSASTKGPS
	.. .**...*.**.***********************.** **********
pH52-8.0	QMNSLRAEDTAVYYCARWRGLNYGFDVRYFDVWGQGTLVTVSSASTKGPS
	110 120 130 140 150

	140 150 160 170 180
H52H4-160	VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
	****** *.*** .***** .*.*********************** *****
pH52-8.0	VFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
	160 170 180 190 200

	190 200 210 220 230
H52H4-160	QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH
	***********.** ..***** ***.********************** **
pH52-8.0	QSSGLYSLSSVVTVTSSNFGTQTYTCNVDHKPSNTKVDKTVERKCC---V
	210 220 230 240

	240 250 260 270 280
H52H4-160	TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK
	****** ..*.******************************.********.
pH52-8.0	ECPPCPAPP-VAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQ
	250 260 270 280 290

FIG. 6A-2

```
              290       300       310       320       330
H52H4-160  FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS
           ******.*.**********.***.*.**.**.*********************
pH52-8.0   FNWYVDGMEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVS
              300       310       320       330       340

              340       350       360       370       380
H52H4-160  NKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYP
           **.*********.*.*************************************
pH52-8.0   NKGLPAPIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYP
              350       360       370       380       390

              390       400       410       420       430
H52H4-160  SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS
           *****************.**.*.*****************************
pH52-8.0   SDIAVEWESNGQPENNYKTTPPMLDSDGSFFLYSKLTVDKSRWQQGNVFS
              400       410       420       430       440

              440       450
H52H4-160  CSVMHEALHNHYTQKSLSLSPGK
           ***********************
pH52-8.0   CSVMHEALHNHYTQKSLSLSPGK
              450       460
```

FIG. 6B

```
                              10        20        30
H52L6-158                     DVQMTQTTSSLSASLGDRVTINCRASQDINN
                              *.****. ******.*********
pHI52-9.0   MGWSCIILFLVATATGVHSDIQMTQSPSSLSASVGDRVTITCRASQDINN
                     10        20        30        40        50

                      40        50        60        70        80
H52L6-158     YLNWYQQKPNGTVKLLIYYTSTLHSGVPSRFSGSGSGTDYSLTISNLDQE
              *.***. .  *************************.*.*.  *
pH52-9.0      YLNWYQQKPGKAPKLLIYYTSTLHSGVPSRFSGSGSGTDYTLTISSLQPE
                     60        70        80        90        100

                      90        100       110       120       130
H52L6-158     DIATYFCQQGNTLPPTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTAS
              *.***.*************************************
pH52-9.0      DFATYYCQQGNTLPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTAS
                     110       120       130       140       150

                      140       150       160       170       180
H52L6-158     VVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTL
              *************************************************
pH52-9.0      VVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTL
                     160       170       180       190       200

                      190       200       210
H52L6-158     SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
              *********************************
pH52-9.0      SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
                     210       220       230
```

9 / 12

SUBSTITUTE SHEET

FIG. 7A-1

```
verA.hcfab1     1 EVQLVESGGGLVQPGGSLRLSCATSGYTFTEYTMHWMRQAPGKGLEWVAG
verI.hcfab2     1 EVQLVESGGGLVQPGGSLRLSCATSGYTFTEYTMHWMRQAPGKGLEWVAG
verN.hcfab3     1 EVQLVESGGGLVQPGGSLRLSCATSGYTFTEYTMHWMRQAPGKGLEWVAG
verO.hcfab4     1 EVQLVESGGGLVQPGGSLRLSCATSGYTFTEYTMHWMRQAPGKGLEWVAG
verO.hcfab25    1 EVQLVESGGGLVQPGGSLRLSCATSGYTFTEYTMHWMRQAPGKGLEWVAG
verP.hcfab6     1 EVQLVESGGGLVQPGGSLRLSCATSGYTFTEYTMHWMRQAPGKGLEWVAG
verQ.hcfab7     1 EVQLVESGGGLVQPGGSLRLSCATSGYTFTEYTMHWMRQAPGKGLEWVAG
verR.hcfab8     1 EVQLVESGGGLVQPGGSLRLSCATSGYTFTEYTMHWMRQAPGKGLEWVAG
verS.hcfab9     1 EVQLQQSGPELVQPGGSLRLSCATSGYTFTEYTMHWMRQAPGKGLEWVAG
verT.hcfab10    1 EVQLVESGGGLVKPGASLRLSCATSGYTFTEYTMHWMRQAPGKGLEWVAG
verU.hcfab11    1 EVQLVESGGGLVQPGGSLKISCKTSGYTFTEYTMHWMRQAPGKGLEWVAG
verV.hcfab12    1 EVQLVESGGGLVQPGGSLRLSCATSGYTFTEYTMHWMKQSHGKSLEWVAG
verW.hcfab13    1 EVQLVESGGGLVQPGGSLRLSCATSGYTFTEYTMHWMRQAPGKGLEWIGG


verA.hcfab     51 INPKNGGTSYADSVKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARWR
verI.hcfab     51 INPKNGGTSHNQRFMDRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARWR
verN.hcfab     51 INPKNGGTSHNQRFMDRFTLAVDKSKNTLYLQMNSLRAEDTAVYYCARWR
verO.hcfab     51 INPKNGGTSHNQRFMDRFTISVDKSTSTAYMQMNSLRAEDTAVYYCARWR
verO.hcfab2    51 INPKNGGTSHNQRFMDRFTISVDKSTSTAYMQMNSLRAEDTAVYYCARWR
verP.hcfab     51 INPKNGGTSHNQRFMDRFTISVDKSKNTLYMELRSLRAEDTAVYYCARWR
verQ.hcfab     51 INPKNGGTSHNQRFMDRFTISVDKSKNTLYLQMNSLTSEDSGIYYCARWR
verR.hcfab     51 INPKNGGTSHNQRFMDRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARWR
verS.hcfab     51 INPKNGGTSHNQRFMDRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARWR
verT.hcfab     51 INPKNGGTSHNQRFMDRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARWR
verU.hcfab     51 INPKNGGTSHNQRFMDRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARWR
verV.hcfab     51 INPKNGGTSHNQRFMDRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARWR
verW.hcfab     51 FNPKNGGTSHNQRFMDRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARWR


verA.hcfab    101 GLNYGFDVRYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALG
verI.hcfab    101 GLNYGFDVRYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALG
verN.hcfab    101 GLNYGFDVRYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALG
verO.hcfab    101 GLNYGFDVRYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALG
verO.hcfab2   101 GLNYGFDVRYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALG
verP.hcfab    101 GLNYGFDVRYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALG
verQ.hcfab    101 GLNYGFDVRYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALG
verR.hcfab    101 GLNYGFDVRYFDVWGAGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALG
verS.hcfab    101 GLNYGFDVRYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALG
verT.hcfab    101 GLNYGFDVRYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALG
verU.hcfab    101 GLNYGFDVRYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALG
verV.hcfab    101 GLNYGFDVRYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALG
verW.hcfab    101 GLNYGFDVRYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALG
```

1	SEQ.ID.NO.26	8	SEQ.ID.NO.33
2	SEQ.ID.NO.27	9	SEQ.ID.NO.34
3	SEQ.ID.NO.28	10	SEQ.ID.NO.35
4	SEQ.ID.NO.29	11	SEQ.ID.NO.36
5	SEQ.ID.NO.30	12	SEQ.ID.NO.37
6	SEQ.ID.NO.31	13	SEQ.ID.NO.38
7	SEQ.ID.NO.32		

FIG. 7A-2

```
verA.hcfab     151 CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
verI.hcfab     151 CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
verN.hcfab     151 CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
verO.hcfab     151 CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
verO.hcfab2    151 CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
verP.hcfab     151 CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
verQ.hcfab     151 CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
verR.hcfab     151 CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
verS.hcfab     151 CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
verT.hcfab     151 CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
verU.hcfab     151 CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
verV.hcfab     151 CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
verW.hcfab     151 CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL


verA.hcfab     201 GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT---
verI.hcfab     201 GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT---
verN.hcfab     201 GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT---
verO.hcfab     201 GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT---
verO.hcfab2    201 GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPP
verP.hcfab     201 GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT---
verQ.hcfab     201 GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT---
verR.hcfab     201 GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT---
verS.hcfab     201 GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT---
verT.hcfab     201 GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT---
verU.hcfab     201 GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT---
verV.hcfab     201 GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT---
verW.hcfab     201 GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT---
```

11 / 12

SUBSTITUTE SHEET

FIG. 7B

```
               10        20        30        40        50
verAlc[1]  DIQMTQSPSSLSASVGDRVTITCRASQDINNYLNWYQQKPGKAPKLLIYY
verZlc[2]  DIQMTQSPSSLSASVGDRVTITCRASQDINNYLNWYQQKPGKAPKLLIYY


               60        70        80        90        100
verAlc     TSTLESGVPSRFSGSGSGTDYTLTISSLQPEDFATYYCQQGNTLPPTFGQ
verZlc     TSTLHSGVPSRFSGSGSGTDYTLTISSLQPEDFATYYCQQGNTLPPTFGQ


               110       120       130       140       150
verAlc     GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV
verZlc     GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV


               160       170       180       190       200
verAlc     DNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG
verZlc     DNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG


               210
verAlc     LSSPVTKSFNRGEC
verZlc     LSSPVTKSFNRGEC
```


1.   (SEQ.ID.NO.39)

2.   (SEQ.ID.NO.40)

12 / 12

	International Application No
	PCT/US 93/07832

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5   C12N15/13   C12P21/08   C07K13/00   C12N5/10   G06F15/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5   C07K   C12N   G06F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF MOLECULAR BIOLOGY vol. 215 , 1990 , ACADEMIC PRESS pages 175 - 182 Tramontano, Anna; Chothia, Cyrus; Lesk, Arthur M. 'Framework residue 71 is a major determinant of the position and conformation of the second hypervariable region in the VH domains of immunoglobulins' cited in the application See the whole document, especially paragraph 7 ---	1-12,15
Y	WO,A,90 07861 (PROTEIN DESIGN LABS, INC.) 26 July 1990 See pages 1-6; 9-25 ---	1-12,15

-/--

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

Date of the actual completion of the international search	Date of mailing of the international search report
22 December 1993	0 1 -02- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Nauche, S

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NATURE. vol. 342 , December 1989 , LONDON GB pages 877 - 883 Chothia, Cyrus; Lesk, Arthur M.; Tramontano, Anna; Levitt, Michael; Smith-Gill, Sandra J.; Air, Gillian; Sheriff, Steven; Padlan, 'Conformations of immunoglobulin hypervariable region' cited in the application See the whole document, especially 'Discussion' ---	1-12,15
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 89 , May 1992 , WASHINGTON US pages 4285 - 4289 Carter, Paul et al. 'Humanization of an anti-p185HER2 antibody for human cancer therapy.' see the whole document ---	1-18
P,X, L	WO,A,92 22653 (GENENTECH, INC.; US) 23 December 1992 see the whole document -----	1-16

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:
   .Claims 17 - 18 (Rule 39.I.vi. PCT) - Program for computers.

2. [ ] Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such
   an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all
   searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment
   of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report
   covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

      [ ] No protest accompanied the payment of additional search fees.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9007861	26-07-90	AU-A-	5153290	13-08-90
		CA-A-	2006865	28-06-90
		EP-A-	0451216	16-10-91
WO-A-9222653	23-12-92	AU-A-	2250992	12-01-93